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(71) Applicant : OJI PAPER COMPANY LIMITED
7-5 Ginza 4-Chome Chuo-ku
Tokyo 104 (JP)

(72) Inventor : Tsukamoto, Akira
1-13-26, Kitashinjuku
Shinjuku-ku, Tokyo (JP)
Inventor : Matsufuji, Mieko
3-2-2, Shiba
Kawaguchi-shi, Saitama (JP)
Inventor : Kita, Yukio
5-11-18, Shimomeguro
Meguro-ku, Tokyo (JP)

(74) Representative : Arthur, Bryan Edward
Withers & Rogers 4 Dyer's Buildings Holborn
London EC1N 2JT (GB)

(54) Ornithine carbamoyl transferase gene and its use as a marker gene in a host-vector system for the production of proteins in basidiomycetes.

(57) This invention relates to a novel ornithine carbamoyl transferase (OCTase) gene, a recombinant DNA containing the OCTase DNA, a transformation system for a basidiomycete, and production of useful proteins using the system.

More particularly, this invention relates to the OCTase gene of Coriolus hirsutus to afford an efficient host-vector system in basidiomycetes (particularly a white rot fungus such as Coriolus hirsutus IFO 4917) for the preparation of useful proteins. OCTase is the enzyme to transform ornithine to citrulline in arginine biosynthesis in organisms. The present invention provides the OCTase genes of C. hirsutus, the useful Arg auxotrophic mutant of C. hirsutus deficient in the OCTase gene, the efficient condition for the preparation of protoplasts and the transformation of the mutant with the cloned OCTase gene and the recombinant DNA's including a promoter, a signal peptide-coding DNA and a protein-coding DNA, the construction of the novel host-vector system of C. hirsutus, and the new method of a large scale preparation of useful proteins using this novel host-vector system with a new recombinant DNA technique.

Furthermore, this invention provides a highly efficient method to produce a useful protein such as lignin peroxidase which is difficult to produce by the conventional method.

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1. Field of the Invention

This invention relates to a novel ornithine carbamoyl transferase (OCTase) gene, a recombinant DNA containing the OCTase DNA, a transformation system for a basidiomycete, and production of useful proteins using the system.

More particularly, this invention relates to the OCTase genes of Coriolus hirsutus to afford an efficient host-vector system in basidiomycetes (particularly a white rot fungus such as C. hirsutus IFO 4917) for the preparation of useful proteins.

2. Description of the Related Art

The OCTase is an enzyme to transform ornithine to citrulline in arginine biosynthesis in organisms. An Arg^- auxotrophic mutant deficient in the aforementioned OCTase gene can be prepared by UV irradiation or N-methyl-N'-nitro-N-nitrosoguanidine (NTG) treatment of C. hirsutus.

The development of the aforementioned OCTase genes of C. hirsutus as the marker, the Arg^- auxotrophic mutant of C. hirsutus as the host, the efficient condition for the preparation of protoplasts and the transformation of the mutant with the cloned OCTase DNA, and the recombinant DNA's including a promoter, a signal peptide-coding DNA and a protein-coding DNA, has realized the construction of the novel host-vector system of C. hirsutus. Thus, this novel host-vector system with a new recombinant DNA technique can provide a new method of a steady, large scale preparation of useful proteins. Furthermore, this invention provides a highly efficient method to produce a useful protein such as lignin peroxidase which is difficult to produce by the conventional method.

To date numerous efforts have been made to fulfill the purpose of realizing quantity production of a useful protein by the use of the technique of gene recombination.

The technique of gene recombination for the quantity production of a useful protein basically comprises a host, a vector and a gene coding a useful protein.

One of the established host which are usable for this purpose is a procaryote such as Escherichia coli. However, the process of the large scale production of useful proteins is not promising very often. For example, in the case of Escherichia coli as the host, it is very difficult to produce a useful human protein without any contamination of harmful byproducts. Therefore, it prevents simplification and economization of the large scale production of the protein.

One of the solution for the aforementioned problem is to use a lower eucaryote such as yeast as the host. However, in this case, the productivity is not acceptable for practical use so far. Thus, efforts are being continued in research and development of a host-vector system capable of more efficient production of useful proteins.

Basidiomycetes belonging to eucaryotes are much phylogenetically closer to animals than yeasts (T.L. Smith, Proc. Natl. Acad. Sci. USA, 86 7063 (1989)). Incidentally, a technique of gene recombination using a basidiomycete as a host remains yet to be established. Recently, a few host-vector systems of basidiomycetes have been reported (A. Munoz-Rivas, et al., Mol. Gen. Genet., 205, 103, (1986)). However, these systems have not been accomplished for practical use so far. The basidiomycetes include numerous useful fungi such as lignin-degrading fungi remains yet to be established. Especially, genetics of C. hirsutus belonging to basidiomycetes has not been studied very well. Furthermore, a host-vector system of this fungus has been hitherto unknown.

SUMMARY OF THE INVENTION

The present inventors have pursued a diligent study with a view to fulfilling the demand for development of an efficient host-vector system for the large scale preparation of useful proteins. Particularly, the demand for development of a novel suitable marker gene, a useful host, an efficient transformation method and condition, and a useful recombinant DNA of a useful protein gene including a promoter and a signal peptide-coding DNA in basidiomycetes.

They have consequently succeeded in developing novel DNA's, i.e. ornithine carbamoyl transferase (OCTase) genes of Coriolus hirsutus and an efficient host-vector system of the genus Coriolus for the expression, secretion and production of useful proteins in a basidiomycete in a large amount.

Accordingly, the present invention provides (1) an isolated structural gene coding for ornithine carbamoyl transferase (OCTase) of Coriolus hirsutus. The gene preferably coding for the amino acid sequence shown in SEQ ID NO: 1 or SEQ ID NO: 2.

The present invention also provides (2) an isolated OCTase gene comprising a structural gene coding for

OCTase and a control region controlling the expression of the structural gene. The OCTase gene, preferably, essentially consists of the nucleotide sequence shown in SEQ ID NO: 4.

The present invention further provides (3) a recombinant linear or circular DNA comprising a structural gene coding for OCTase of C. hirsutus, or an OCTase gene comprising a structural gene coding for OCTase of C. hirsutus and a control sequence controlling the expression of the OCTase. The recombinant DNA provides a selectable marker showing successful transformation when a host basidiomycete is cotransformed with a DNA comprising a gene coding for a desired protein.

The present invention further provides (4) a recombinant linear or circular DNA comprising:

(a) a structural gene coding for OCTase of C. hirsutus, or an OCTase gene comprising a structural gene coding for OCTase of C. hirsutus and a control sequence for controlling the expression of the structural gene;

(b) an expression control sequence operable in a basidiomycete of the genus Coriolus; and

(c) a gene coding for a desired protein under the control by the expression control sequence (b). The circular DNA is, for example, an expression plasmid for expressing the desired protein.

The present invention also provides (5) an auxotrophic mutant of a basidiomycete of the genus Coriolus deficient in an ability to express OCTase. The auxotrophic mutant is a partner of a host-vector system of the present invention.

Namely, the present invention further provides (6) a host-vector system for basidiomycete comprising the recombinant liner or circular gene (3) or (4), and an auxotrophic mutant (5).

The present invention still more provides (7) a process for production of a transformant basidiomycete of the genus Coriolus capable of expression of a desired protein comprising the steps of (a) cotransforming the abovementioned auxotrophic mutant (5) with the said recombinant linear or circular DNA (3) and a recombinant linear or circular DNA comprising a gene coding for a desired protein and a control sequence for controlling the expression of the gene coding for the desired protein, and (b) selecting a transformant capable of growing a medium lacking arginine.

The present invention further provides (8) a process for production of a transformant basidiomycete of the genus Coriolus, comprising the steps of (a) transforming the auxotrophic mutant (5) with the recombinant linear or circular DNA (4).

The present invention further provides a transformant basidiomycete obtainable by the process (8) capable producing a desired protein.

The present invention still more provides (9) a process for production of a desired protein, comprising the steps of culturing a transformant basidiomycete (8) to produce the desired protein, and recovering the desired protein from the culture.

BRIEF DESCRIPTION OF THE FIGURES

Fig.1 is a restriction endonuclease physical map of plasmid, pUCR1.
Fig. 2 is the construct ion of a expression vector, pPC1 described in example 8.
Fig.3 is the construction of a lignin peroxidase expression vector, pPgHLC1 described in example 8.
Fig.4 is the construction of a lignin peroxidase expression vector, pPcHLC1, described in example 9.
Fig 5 is the construction of a lignin peroxidase expression vector pPSgHL1 described in example 10.
Fig.6 is the construction of a lignin peroxidase expression vector pPSproHL1 described in example 11.
Fig. 7 is the construction of a lignin peroxidase expression vector pRPgHL1 described in example 12.
Fig 8 is the construction of a fragment of OCTase gene described in example 13.
Fig 9 is the recombinant DNA pRPC1 described in example 13.
Fig.10 is the construction of a lignin peroxidase expression vector pRPgHLC1 described in example 13.
Fig.11 is the construction of a phenoloxidase expression vector pRPgPO1 described in example 16.
Fig.12 is the construction of a luciferase expression vector pPLUCC1 described in example 19.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present inventors have studied OCTase gene of Coriolus hirsutus to find an efficient host-vector system in basidiomycetes for the preparation of useful proteins. They have succeeded in the cloning of novel OCTase genes from the restriction endonuclease fragments of chromosome DNA of C. hirsutus. They have also prepared a useful Arg⁻ auxotrophic mutant deficient in OCTase gene from C. hirsutus and established the efficient condition for the preparation of protoplasts and transformation of the mutant with the cloned OCTase DNA and recombinant DNA's including a promoter, a signal peptide-coding DNA and a protein-coding DNA. Thus they have succeeded in the construction of the novel host-vector system of C. hirsutus. Furthermore,

they have found that this novel host-vector system using recombinant DNA technique can provide a new method of a robust, large scale production of useful proteins.

The present invention provides a DNA (I), concerning an ornithine carbamoyl transferase (OCTase) gene coding the amino acid sequence depicted in SEQ. ID NO: 1.

The present invention also provides a DNA (II), concerning an ornithine carbamoyl transferase (OCTase) gene coding the amino acid sequence depicted in SEQ ID NO: 2.

The present invention also provides a DNA (III), concerning an OCTase gene coding the DNA sequence depicted in SEQ ID NO: 3.

The present invention also provides a genomic DNA (IV), concerning an OCTase gene coding the DNA sequence depicted in SEQ ID NO: 4.

The present invention also provides a method to prepare a novel organism (particularly a white rot fungus Coriolus hirsutus) possessing the ability to produce a useful protein by the transformation with any of DNA (I) to (IV) and/or a recombinant DNA consisting of a functional promoter in basidiomycetes and/or a signal-peptide-coding DNA and a protein-coding DNA.

The present invention also provides a recombinant DNA as described above, consisting of a functional promoter in Coriolus species and/or a signal-peptide-coding DNA and a protein-coding DNA.

The present invention also provides the aforementioned recombinant DNA, wherein said promoter is a promoter of phenoloxidase gene, a promoter of lignin peroxidase gene or OCTase gene, and/or said signal-peptide-DNA is coding a signal peptide of phenoloxidase or a signal peptide of lignin peroxidase.

The present invention also provides a method to prepare a novel organism as described above, wherein the host cell is deficient in OCTase and the tarnsformant is screened in a medium containing no arginine.

The present invention also provides the aforementioned novel organism, wherein said organism is Coriolus species.

The present invention also provides a method to produce a protein as described above, wherein said protein is produced by culturing said cell and obtaining proteins from the resultant culture broth.

The aforementioned basidiomycete used in the cloning of genomic DNA and mRNA was a strain of C. hirsutus (IFO 4917) obtained from the culture collection of the Institute for Fermentation, Osaka, Japan. A method to extract total RNA and to construct a cDNA library is as described below.

Total RNA was extracted from the mycelia of C. hirsutus cultured in a medium containing no arginine to induce the expression of OCTase gene. Poly(A) RNA was purified from the total RNA according to the method described in Proc. Natl. Acad. Sci. USA, 69, 1408 (1972), using oligo(dT) cellulose column. cDNA was synthesized in vitro from the poly(A) RNA according to the method described in Gene, 25 263 (1983), incorporated in a phage DNA λgt according to the method described in Science, 222, 263 (1983), and packaged in vitro to afford the cDNA library for the OCTase gene cloning.

To conduct plaque hybridization for the cloning of the OCTase gene from the aforementioned cDNA library, the synthetic DNA probes synthesized based on the DNA sequences of the OCTase genes isolated from other species (B. Berse, et al., Gene, 25, 109 (1983); F. P. Buxton, et al., Gene 60, 255 (1987) can be used. In addition, the aforementioned hybridization can be also conducted using a fragment of the Arg B gene coding the OCTase gene of Aspergillus nidulans (Upshall,A., M.G.G., 204, 349 (1986)). The isolated DNA can be sequenced by the method described by Sanger (Proc. Natl. Acad. Sci. USA, 74, 5463, (1977)). The transformed E. coli JM-109/pLCRM harboring the recombinant cDNA of OCTase gene was deposited with National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology under FERM BP-4202.

Chromosome DNA can be purified from C. hirsutus using the conventional method such as described by Yelton (Proc. Natl. Acad. Sci. USA, 81, 1470 (1984)). The genomic DNA obtained was then partially digested with an appropriate restriction endonuclease, followed by a fractionation using a sucrose density gradient centrifugal separation to afford a 10 to 25 kbp genomic DNA fragment. The resultant DNA fragment was inserted to the phage DNA treated with an appropriate restriction endonuclease. For example, DNA EMBL3 (A-M, Frisnauf, et al., J. Mol. Biol. 170, 827 (1983)) can be used as the aforementioned phage. The reconbinant phage DNA was packaged in vitro to construct a genomic DNA library. For the subcloning of the DNA can be used a conventional cloning vector, such as pUC13 (C. Yanisch-Perron, et al., Gene, 33, 103 (1985)).

The aforementioned OCTase gene cloned from the cDNA library can be used for the probe to isolate the genomic OCTase gene from the genomic library as described above. The restriction endonuclease physical map is depicted in Fig. 5. The genomic OCTase gene can be inserted into an appropriate E. coli cloning vector, such as pUC type cloning vector, pUC18 to transform an Arg$^-$ auxotrophic mutant of Coriolus species such as C. hirsutus mutant. auxotrophic mutants of Coriolus species such as C. hirsutus can be prepared using the conventional method as described below.

Monokaryons of C. hirsutus can be prepared from colonies of mycelial protoplast regenerants as well as germinated basidiospores. Oidia formed from the monokaryons can be then subjected to irradiate with UV or

treated with chemical mutagens, such as N-methyl- N'-nitro-N-nitrosoguanidine (NTG) or ethyl methanesulfonate. The mutant can be characterized in detail as described in example 5 to select the amino acid requiring mutants. In example 6, the deficiency in the amino acid biosynthetic pathway can be further determined.

Thus, the novel host-vector system of an eucaryote, C. hirsutus can be provided by the reconbinant vector DNA containing the cloned OCTase gene of C. hirsutus as well as the Arg^- auxotrophic mutant of C. hirsutus. The transformed Arg^- auxotrophic mutant of C. hirsutus, introduced the aforementioned OCTase gene, can produce the enzyme which was deficient in the mutant. Therefore, the aforementioned OCTase gene of C. hirsutus contains the complete open reading frame (the protein-coding region) of the OCTase gene as well as the regulation region necessary for the expression of the OCTase gene. This conclusion can be confirmed by the existence of the promoter sequence in the upstream region of the DNA sequence of the genomic OCTase gene.

The Arg^- auxotrophic mutant of C. hirsutus, OJI-1078 and the E. coli JM109/pUCR1 transformed with the aforementioned genomic OCTase gene were deposited with National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology under FERM P-12677 and FERM BP-4201, respectively.

The present invention further provides a recombinant DNA for the novel host-vector system as well as a new method of a large scale production of useful proteins.

This reconbinant DNA consists of a protein-coding DNA as well as a functional promoter in basidiomycetes and/or a signal-peptide-coding DNA at the 5' terminus of the protein-coding DNA.

This promoter used for the expression of the protein can be a promoter functional promoter in Coriolus species such as C. hirsutus. An efficient expression of the protein can be expected using the promoter of C. hirsutus, such as the promoter of phenoloxidase (Y. Kojima, et al., J. Biol. Chem., 265, 25, 15224 (1990)). Furthermore, the promoter of lignin peroxidase can be used for the same purpose, which was described in the Japanese Patent Aplication 92-60503 and also deposited as a transformed E. coli XL-1 blue/pBSLPOG7 harboring a plasmid pBSLPOG7 containing the aforementioned promoter with National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology under FERM P-12683. In addition, a promoter of another lignin peroxidase gene can be also used for the same purpose, which was described in the Japanese Patent Aplication 92-52673 and also deposited as a transformed E. coli JM109/pUCLPOG4 harboring a plasmid pUCLPOG4 containing the aforementioned promoter with National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology under FERM P-12678.

For the aforementioned signal-peptide-coding DNA can be used any functional signal-peptide-coding DNA of Coriolus species such as C. hirsutus i.e. the original promoter of a useful protein a signal-peptide-coding DNA of yeast or filamentous fungi. Moreover, a synthetic DNA designed for the signal-peptide-coding DNA can be also used. These signal sequences as described above, can be a functional signal-peptice-coding DNA of C. hirsutus for secretionary expression, such as a signal peptide of phenoloxidase or a signal peptide of lignin peroxidase.

A useful protein which can be expressed and produced in basidiomycete, C. hirsutus are enzymes, growth factors, hormones, cytokinins, and virus proteins such as, for example human lisozymes, protein disulfide isomerases (PDI), human epidermal growth factors (EGF), human nerve growth factors (NGF), growth hormones, insulins, interferon α, interferon β, interferon γ, interleukin 2, tumor necrosis factors (TNF), surface proteins of B type hepatitis, lymphotoxins, lignin peroxidases, and phenoloxidases.

The DNA sequences coding the aforementioned useful proteins can be complementary DNA's (cDNA), genomic DNA's or synthetic DNA's.

In the case of the existence of a propeptide between the signal peptide and the protein-coding sequence, the whole DNA sequence including the propeptide sequence can be used for the expression of the protein. On the contrary, in the case of a protein containing no propeptide, a propeptide DNA sequence can be inserted between the signal sequence and the protein-coding sequence.

In the case of the production of lignin peroxidase with a wild strain of C. hirsutus, a restricted condition such as low nitrogen content of the medium, high concentration of oxygen, statical culture is required preventing efficient large scale production of the enzyme. Cloning's of the gene of lignin peroxidases have been reported only for Phanerochaete chrysosporium (Nature, 326, 520, (1987)), Coriolus versicolor (B.B.R.C., 179, 428 (1991)), and Phlebia radiata (Gene, 85, 343 (1989). However, no active enzyme has been produced using the conventional method.

By using the present host-vector system of C. hirsutus active lignin peroxidase can be produced efficiently using any lignin peroxidase gene, such as a genomic DNA, cDNA and a synthetic DNA.

DNA introduced for the transformation of C. hirsutus can be any functional marker gene in Coriolus species such as C. hirsutus, for example the reconbinant vector DNA containing the OCTase gene. The suitable vectors which can be used in the system can be any vector DNA capable of amplifying the recombinant DNA in a host such as E. coli for a convenient gene manipulation, for example pUC vector, pBR322 vector or pBluescript vector. The aforementioned plasmid vector can be used as a selection marker gene as a circular form DNA and

a linear form DNA as well as the OCTase gene fragment.

An expression vector of a protein such as lignin peroxidase can be constructed by the insertion of a promoter DNA ligated the structural gene of the protein at the downstream region into the aforementioned recombinant DNA. Furthermore, more efficient expression can be obtained by the insertion of a functional terminator sequence of Coriolus species such as C. hirsutus at the downstream region of the protein-coding sequence such as lignin peroxidase gene.

Moreover, the aforementioned OCTase DNA and the expression vector of a protein can be co-transformed without ligating each other as a circular, linear or fragment form to give the transformants.

The method to construct the aforementioned expression vector can be the conventional method described by Sambrook et al.(Molecular Cloning A Laboratory Manual / 2nd Ed.).

Examples of the expression plasmid of the lignin peroxidase are such as pPgHLC1, pPcHLC1, pPSgHL1, pPSproHL1, pRPgHL1, and pRPgHLC1 as described in examples 8, 9, 10, 11, 12, and 13, respectively.

Coriolus is species such as C. hirsutus can be transformed using those expression vectors. The transformation of protoplasts of C. hirsutus prepared by the method described in example 7 can be conducted by the polyethylene glycol method and the electroporation method. The preferred concentration of the polyethylene glycol for the polyethylene glycol method is in a range of 10% to 50%. The preferred concentration of calcium ion is in a range of 25 mM to 200mM.

The resultant transformants can be cultured by the conventional method. For example, the transformant can be cultured in gulucose-peptone medium at 15 to 40°C (preferentially at 24 to 37°C) for 7 days with or without shaking. Air bubbling or agitation can be used if necessary.

After completion of the culture the culture broth can be separated from the cell bodies by the conventional method. When the product is contained in the cell, the cell bodies can be homogenized by the supersonic method, the frenchpress method, the mechanical grinding method, and the enzymatic lysis method. Optionally, the chemical method using surfactants such as triton-X100, deoxycholate can be combined to the aforementioned method. The resultant culture broth and the extracts of the cell bodies can be purified using the conventional protein purification methods, such as salt precipitation, isoelectric precipitation, gel filtration, and ion-exchange chromatography (HPLC, FPLC etc.), to obtain the product.

The activity of the lignin peroxidase obtained from the aforementioned method can be measured by the increasing absorbance of veratraldehyde produced by the oxidation of veratryl alcohol (M. Tien, et al., Proc. Natl. Acad. Sci. USA, 81, 2280 (1984))

EXAMPLES

Now, cloning of a novel OCTase gene of C. hirsutus, preparation of a useful host C. hirsutus, development of an efficient transformation method and condition, and construction of a useful recombinant DNA of a useful protein gene including a promoter and a signal peptide-coding DNA will be described in detail below with reference to working examples. It should be noted, however, that this invention is not limited to these working examples.

Example 1: Construction of cDNA library

A wild-type dikaryotic strain of C. hirsutus Quele (IFO 4917) was obtained from the culture collection of the Institute for Fermentation, Osaka, Japan. C. hirsutus is a species in which the basidiospore is mononucleate, and the parental nuclei do not fuse after mating of two compatible monokaryons but stay in pairs(Bose 1934). monokaryons of C. hirsutus were isolated from colonies of mycelial protoplast regenerants as well as germinated basidiospores obtained from a fruit body of the dikaryotic cultures. Vegetative cultures were maintained on slants of potato-dextrose medium.

The culture media used in this invention are such as MM medium, SMY medium, GP medium, synthetic medium as described below.

The MM (minimal medium), pH 5.6, containing per liter 10g glucose, 1.5g $(NH_4)_2HPO_4$, 1g K_2HPO_4, 0.5g KH_2PO_4, 1.5g $MgSO_4$-$7H_2O$, 0.12mg thiamine-HCl.

The SMY medium, pH 5.6, containing per liter10g sucrose, 20g malt extract, 4g yeast extract.

The GP medium, pH 4.5, containing per litter 20 g of glucose, 5 g of polypeptone, 2 g of yeast extract, 1 g of K_2HPO_4, and 0.5 g of $MgSO_4$ $7H_2O$

The synthetic medium, pH5.6, containing no arginine, 2% of glucose, 0.67% of Yeast Nitrogen Base (Difco), 0.1% of K_2HPO_4, 0.15% of $(NH_4)_2HPO_4$, 0.05% of KH_2PO_4, 0.15% of $MgSO_4$ $7H_2O$, 0.12ppm of thiamine HCl, 40ppm of glycine, 40ppm of alanine, 60ppm of valine, 70ppm of leucine, 70ppm of isoleucine, 50ppm of serine, 60ppm of threonine, 60ppm of proline, 80ppm of phenylalanine, 90ppm of tyrosine, 100ppm of tryptophan,

70ppm of lysine·HCl, 80ppm of histidine·HCl, 120ppm of cysteine·HCl, 70ppm of methionine, 70ppm of aspartic acid, and 90ppm of glutamic acid.

In 200 ml of a GP medium Coriolus hirsutus IFO 4917 was shaken-cultured at 28°C for 7 days. Then the cells were collected and washed with sterilized water. The cells were further shaken-cultured at 28°C for 4 days in 500 ml of a synthetic medium. The cells were collected and frozen in liquefied nitrogen.

Five gram of the frozen cell bodies was ground with a mortar. The apparatuses and reagents used in the following experiment were treated with diethylpyrocarbonate according to the conventional method (Sambrook, Molecular Cloning A Laboratory Manual 2nd Ed., 1989). The extraction of the ground cell bodies was carried out by the use of a commercially available RNA extraction kit (produced by Amersham Japan K.K.) to afford total RNA. Two milligram of the total RNA was purified using a commercially available mRNA purification kit (produced by Pharmacia) to give poly(A)$^+$RNA fraction. From 5 μg of the poly(A)$^+$RNA, double-stranded cDNA was synthesized using a commercially available cDNA synthesizing kit (produced by Amersham Japan K.K.) according to the manual. cDNA library was constructed using a commercially available λ gt10 cDNA cloning kit (produced by Amersham Japan K.K.) according to the manual to obtain 5 x10^5 recombinant λ phage bodies/ μg insert DNA. The cDNA library was caused to infect an E. coli NM514 strain to form 2,000-3,000 plaques / 90mmϕ plate for a cloning experiment.

Example 2: Cloning of OCTase gene from cDNA library

The cloning of the OCTase gene was conducted according to the conventional plaque hybridization method (Sambrook, Molecular Cloning A Laboratory Manual 2nd Ed., 1989).

The following two synthetic DNA probes labeled with a radioisotope (^{32}P) were used in the hybridization experiment.

(A) 5'-TTT(C)ATGCAT(C)TGT(C)CTICC-3' 17mer, 8mix

(B) 5'-CCA(G)TAA(G)AAA(G,C,T)ACC(T)TCA(G)TC-3' 17mer, 64mix

In addition, 0.8 kbp fragment of Sal I digest of Arg B gene of Aspergillus nidulans labeled with ^{32}P was used for the same experiment.

The plaques were transferred to a commercially available nylon membrane to immobilize the DNA on the membrane by the conventional method. The membrane was washed with a pre-hybridization solution (5 x SSC, 5 x Denhardt's solution, 1% SDS, and 100 μg/ml denatured DNA calf thymus DNA) at 65°C overnight. The membrane was then hybridized with the hybridization solution containing ^{32}P labeled DNA probe at 45°C for 24hr. The membrane was then washed, dried by the conventional method. The positive plaque was selected from the membrane by autoradiography to give a single positive clone hybridized with the aforementioned three DNA probes from 80,000 plaques.

The phage DNA was prepared from the positive clone by the conventional method, followed by the digestion with restriction endonuclease Bam HI and the fractionation using agarose gel electrophoresis to obtain 1.3 kbp of Bam HI DNA fragment. The DNA fragment was then subcloned into the Bam HI site of a plasmid vector pUC18 (produced by Takara Shuzo Co., Ltd. Japan) and transformed to E. coli JM109 by the conventional method. The DNA sequence of the subcloned DNA was then determined using a sequenase kit produced by United States Biochemical Co., Ltd.

The DNA sequence was depicted in SEQ ID NO: 3 to demonstrate 1125 bp of open reading frame. The deduced amino acid sequence from the DNA sequence was then compared with the amino acid sequences of other OCTase such as Arg B of A. nidulans to indicate similarity among them and the consensus sequence for the carbamoyl binding region of OCTase. Therefore, the 1.3 kbp DNA fragment was identified as the OCTase gene of C. hirsutus. This DNA fragment was used as the probe for the cloning of the genomic OCTase gene of C. hirsutus as described in examples 3 and 4.

The aforementioned E. coli JM109/pUCRM transformed with the aforementioned OCTase cDNA gene was deposited with National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology as FERN P-13424 on February 12, 1993, and transferred to an international deposition under the Budapest Treaty, as FERM BP-4202 on February 22, 1993.

Example 3: Construction of genomic DNA library

C. hirsutus IFO4917 was shaken-cultured at 28°C for 7 days in GP medium as described in example 1. The cells were collected, washed and frozen with liquefied nitrogen. Five g of the frozen cell bodies was ground

with a mortar. The ground cell bodies was then mixed with 10ml of a lysis buffer solution (100mM Tris pH8, 100mM EDTA, 100mM NaCl, 100 μg/ml of proteinase K) and incubated at 55°C for 3hrs. The resultant mixture was then extracted with phenol then with chloroform. To the aqueous layer was added ethanol gradually to precipitate pure genomic DNA. The DNA was collected and dissolved in a TE solution.

Hundred μg of the purified genomic DNA was partially digested with a restriction endonuclease Sau 3 AI. The resultant fragments were subjected to a 5 to 25 % sucrose density gradient centrifugal separation (30,000rpm, 18 hr.) to obtain a 10 to 25 kbp DNA fragment. The fragment was then ligated to a Bam HI arm of phage λ EMBL 3 (produced by Toyobo Co., Ltd. Japan). The λ phage DNA was packaged in vitro using a Gigapack Gold kit (produced by Stratagene) and infected to E. coli P2392 to afford the genomic DNA library of C. hirsutus.

Example 4: Cloning of OCTase gene from genomic DNA library

The cloning of the genomic OCTase gene from the aforementioned genomic DNA library was conducted according to the conventional plaque hybridization method (Sambrook, Molecular Cloning A Laboratory Manual 2nd Ed., 1989).

The cDNA fragment cloned in example 2 was used as the probes after labeling with a radioisotope (^{32}P) to select a positive clone. The cloning method was essentially same as described in example 1. The positive plaque was selected from the library by autoradiography to give six positive clones from 40,000 plaques. The phage DNA's were prepared from the positive clones by the conventional method, followed by the digestion with various restriction endonucleases and the Southern hybridization using the aforementioned 1.3kbp cDNA probe to indicate the 5.5kbp fragment of the restriction endonuclease Sal I digests was the positive DNA fragment.

The aforementioned 5.5 kbp Sal I fragment was then subcloned into the Sal I site of a plasmid vector pUC18 and transferred to E. coli JM109 by the conventional method. The DNA sequence of the subcloned DNA was then determined using a sequenase kit produced by United States Biochemical Co., Ltd.

The DNA sequence was depicted in SEQ ID NO: 4 to demonstrate that 4 introns interrupted the genomic DNA. At the upstream region of the translation initiation site (653bp) several TATA-box like sequences, CAAT-box like sequences and cis-acting sequence related to the amino acid biosynthesis can be identified. The comparison of the open reading frame of the genomic DNA with the OCTase cDNA sequence (consisting of 1125 bp of open reading frame) indicated 21 base changes between two OCTase genes. Only one amino acid substitution (Arg to Lys) was identified between the two OCTase genes since 20 of 21 base changes occur at the third position of the triplet codon, not affecting the deduced amino acids. However, the amino acid substitution could not affect the property of the enzyme since both amino acids are a basic amino acid. Therefore, these genes appear to be allelic genes each other.

The aforementioned E. coli JM109/pUCR1 transformed with the aforementioned OCTase cDNA gene was deposited with National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology as FERM P-12679 on December 25, 1991, and transferred to an international deposition under the Budapest Treaty as FERM BP-4201 on February 22, 1993.

Example 5: Preparation of auxotrophic mutants

To induce fruit boby formation, C. hirsutus (IFO 4917) was grown for eight weeks in a solid medium containing 20g of thermomechanical pulp of radiata pine supplemented with 130ml of PD medium per 1 liter Erlenmeyer flask. The cultures were incubated at 28°C and continuously illuminated using a 40W fluorescent lamp at a distance of 1m. C. hirsutus formed a typical fruit boby under the culture condition described above.

Basidiospores were washed from gills with distilled water, filtered through 60 μm pore nylon cloth, diluted appropriately and plated onto PD agar medium. Monokaryons were screened under the microscope for mononucleate and clamp-lacking mycelia. The mating-type of each monokaryon was determined by means of confronting cultures between combinations of two monokaryons on SMY plate.

For oidia formation, a monokaryon was incubated on agar (1.3%) plates of MM supplemented with 1% casamino acid(Difco Laboratories, USA), pH 5.6, for 7-10 days at 28°C. Oidia were collected by rinsing the mycelial turf with distilled water, filtered through 60 μm pore nylon cloth and pelleted by centrifugation at 1,500 x g for 10 min at room temperature.

The oidia (2 x 10^6/ml) in 2ml of phosphate buffer (10mM, pH 7) were subjected with 50 μg/ml of N-methyl-N'-nitro-N-nitrosoguanidine (NTG) at 28°C for 60 min with gentle stirring. This dose of NTG gave 3-5% survival of oidia in a series of mutagenesis treatment. The mutagenized oidia were washed three times with 10ml of phosphate buffer by centrifugation (1,500 x g, 10 min) and plated onto the MM agar and incubated at 28°C.

After 2 days incubation, macroscopically visible colonies which grew on the MM agar plate were marked as prototrophs. Then 5ml of molten soft agar(1%, kept at 45-48°C) of MM supplemented with 1% casamino acid was overlaid onto the MM agar plate and further incubated. Colonies which grew on the overlay of complete medium, but not on the MM were screened as auxotrophic mutants.

To select amino acid requiring mutants, the auxotrophic mutants obtained were tested for growth on MM supplemented with one of five groups of L-amino acids, each at 150 μg/ml. Colonies formed on these media were then transferred onto plates of MM containing individual amino acids or amino acid precursors (150 μg/ml) to identify specific supplement required. Thus, Arg^- auxotrophic mutant was screened. Furthermore, a double-auxotrophic mutant (Arg^-, Leu^-) was prepared by NTG treatment of the Arg^- auxotrophic mutant. These auxotrophic mutants were used as the recipient strain in the following transformation experiment.

Example 6: Characterization of the double-auxotrophic mutant

The aforementioned mutant was studied to characterize the deficient gene in the arginine biosynthesis by the following method.

The mutant was cultured in the following five different media.

(1) MM + 150 μg/ml leucine + 200 μg/ml citrulline
(2) MM + 150 μg/ml leucine + 200 μg/ml ornithine
(3) MM + 150 μg/ml leucine + 200 μg/ml arginine
(4) MM + 150 μg/ml leucine
(5) MM + 0.2% casamino acid

After 48 hr incubation at 28°C, the mutant in the media (1), (3) and (5) appeared to grow, whereas the mutant in the media (2) and (4) did not. Therefore, it was identified that the mutant was deficient in the ornithine carbamoyl transferase (OCTase) gene.

The aforementioned mutant (OJI-1078) is deposited with National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology as FERM P-12677 on December 25, 1991, and transferred to an international deposition under the Budapest Treaty as EERM BP-4210, on March 1 1993.

Example 7: Transformation of Coriolus hirsutus

7-a:

For mycelial protoplast preparation, mycelia (C. hirsutus OJI-1078) were grown in 500ml Erlenmeyer flasks containing 100ml of SMY medium equipped with thirty glass-beads (6mm in diameter) in a flask. The flasks were incubated statically for 7 days, while the flasks were stirred with gentle hand-shaking once or twice a day to prevent mycelia from forming pellets and aerial growth. Then the mycelia were transferred to 1 L Erlenmeyer flasks equipped with magnetic stir-bar, and cultured in 200ml of SMY medium.

7-b:

Mycelia were harvested by suction filtration on 30 μm pore nylon cloth, and washed with osmotic medium (OM,0.5M $MgSO_4$/50mM maleate buffer, pH 5.6). Mycelia(100mg wet weight) suspended in 1ml of OM were treated with 10mg/ml Novozyme 234 (Novo Nordisk Bioindustry Ltd., Japan) and 5mg/ml Cellulase Onozuka R10 (Yakult Honsha, Japan) at 28°C with gentle shaking for 4hrs.

7-c:

Protoplasts were separated from mycelial debris by filtering through 30 μm pore nylon cloth. Mycelial debris and remaining protoplasts on nylon cloth were washed once by pouring OM to release protoplasts. Protoplasts were pelleted by centrifugation and suspended in 4ml of 1M sucrose/20mM MOPS buffer, pH 6.3(SM), washed twice with SM. The protoplasts were then resuspended in SM supplemented with 40mM $CaCl_2$ (SMC) to give 10^7-10^8 cells/ml and reserved at 4°C.

The number of protoplasts in suspension was determined by direct count in a hemacytometer. All centrifugations were at 1,000 x g for 5 min with swinging buckets at room temperature.

For oidial protoplast preparation, 10^8 cells of ungerminated oidia in 1ml of OM were treated as the same procedure for mycelial protoplast preparation as described above except that the nylon cloth filtration step was omitted. Protoplast viability ranged of 5-15%.

7-d :

The aforementioned plasmid pUCR1 was digested with restriction enzyme Sal I. Then the DNA fragments (2 μg in 20 μl of 10mM Tris/1mM EDTA[TE], pH 8.0) were added to the protoplast suspension(10^6 cells/100 μl of SMC), mixed gently, and incubated on ice for 30 min.

The protoplasts suspension were gently mixed with 2/3 volume of PEG solution (25% PEG (M_r=3,400 Aldrich Chemical Co., USA) buffered with 20mM MOPS (pH6.4)) and incubated for 30 min on ice.

The resultant protoplast solution was filled to 10 ml with MM containing 0.5M sucrose, 150 μg/ml leucine and 1% agar, poured over plates, and incubated at 28°C for several days.

The transformants were obtained at a frequency of 300 colonies/μg DNA.

Similarly, using the circular plasmid pUCR1, the transformants were obtained at the same frequency. On the contrary, in the control experiment using pUC18 plasmid vector, no transformant was obtained.

Example 8: Construction expression vector of lignin peroxidase (1)

The expression vector of lignin peroxidase of C. hirsutus was constructed by the ligation of the protein-coding sequence of the genomic lignin peroxidase (Japanese Patent Application 92-60503, pBSLPOG7, National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology under FERM P-12683) to the downstream of the promoter region of the genomic phenoloxidase (Japanese Patent Application 91-15392, FERM BP-2793) as well as the upstream of the terminator region of the genomic phenoloxidase.

Precisely speaking, 5.5 kbp DNA fragment of Hin dIII digests of the phenoloxidase genomic DNA was treated with exonuclease III and mung bean nuclease to remove the protein-coding region of the phenoloxidase followed by the ligation with T4DNA ligase to obtain a 2.0 kbp size plasmid pP1 containing the promoter region of the phenoloxidase (Fig. 2).

On the other hand, the aforementioned plasmid containing the genomic phenoloxidase gene was doubly digested with Bam HI and Sph I, followed by the treatment with exonuclease III and mung bean nuclease, then the ligation with T4DNA ligase, and further digestion with Hin dIII to obtain a 600 bp size fragment containing the size terminator region of the phenoloxidase. This fragment was then inserted into the Bam HI-Sac I site of the aforementioned plasmid pP1 to obtain the plasmid pPC1 (Fig 2).

Furthermore, the recombinant DNA pBSLPOG7 containing the genomic lignin peroxidase gene (Japanese Patent Aplication 92-60503) of C. hirsutus at the Bam HI - Eco RI site of a E. coli. vector pBluescript SK⁻ was treated with exonuclease III and mung bean nuclease to remove the promoter region of the lignin peroxidase followed by the ligation with Sal I linker and digestion with Sal I to obtain the protein-coding region of the lignin peroxidase.

Finally, the plasmid pPC1 containing the promoter region and the terminator region of the phenoloxidase was digested with Sal I followed by the insertion of the aforementioned 1.8kbp lignin peroxidase-coding fragment to obtain the plasmid pPgnLC1 (Fig 3)

Example 9: Construction expression vector of lignin peroxidase (2)

The recombinant DNA pBSLPOC7 (FERM P-12680) constructed by the subcloning of the cDNA gene (Japanese Patent Application 92-60503) of lignin peroxidase of C. hirsutus into the Bam HI site of a E. coli vector pBluescript SK⁻ was digested with Nco I and treated with mung bean nuclease, followed by ligation to Bam HI linker with T4DNA ligase. The resultant DNA was digested with Bam HI to obtain 1.3 kbp fragment of the cDNA of the lignin peroxidase.

On the other hand, the plasmid pPC1 prepared in example 8 was digested with Bam HI and thereafter ligated with the aforementioned 1.3 kbp fragment of the cDNA of the lignin peroxidase to obtain the plasmid pPcHLC1.

Example 10: Construction expression vector of lignin peroxidase (3)

The 5.5 kbp Hin dIII fragment of the phenoloxidase gene (Japanese Patent Application 3-15392, FERM P-12683) was ligated with T4DNA ligase to the Hin dIII site of phage vector M13mp19 to prepare a single-stranded phage DNA. The complement DNA of the signal-peptide-coding science of the phenoloxidase (depicted in Fig. 5), consisting of 21 amino acids, was chemically synthesized using a DNA synthesizer. This DNA primer was annealed to the aforementioned single-strand phage DNA to obtain the DNA fragment of the promoter sequence and the signal-coding sequence using the primer extension method. The resultant DNA was

digested with Hin dIII to obtain 2.1 kbp DNA fragment (Fragment 1).

On the other hand, to obtain the protein-coding DNA sequence of the lignin peroxidase, the plasmid pBSLPOG7 (Japanese Patent Application 92-60503, FERM P-12683) was treated by the essentially same method as described above (depicted in Fig. 5). Thus, the primer extension method and digestion with Sal I to obtain 1.7 kbp DNA fragment (Fragment 2).

The aforementioned two fragments 1 and 2 were then ligated with T4DNA ligase to the E. coli vector pUC18 doubly digested with Hin dIII and Sal I to obtain the plaid pPSgHL1 containing both fragments (depicted in Fig. 5).

Example 11: Construction expression vector of lignin peroxidase (4)

To obtain the propeptide-containing DNA sequence of the lignin peroxidase the plasmid pBSLPOG7 (Japanese Patent Application 92-60503, FERM P-12683) was treated by the essentially same method as described above (depicted in Fig. 6). Thus, the primer extension method and digestion with Sal I to obtain 1.7 kbp DNA fragment (Fragment 3).

The aforementioned two fragments 1 and 3 were then ligated with T4DNA ligase to the E. coli vector pUC18 doubly digested with Hin dIII and Sal I to obtain the plasmid pBSproHL1 containing both fragments (depicted in Fig. 6).

Example 12: Construction expression vector of lignin peroxidase (5)

The expression vector pRPgHL1 of lignin peroxidase of C. hirsutus was constructed by the ligation of the protein-coding sequence of the lignin peroxidase to the downstream of the promote sequence of the OCTase gene of C. hirsutus

In detail, the promoter fragment was prepared by the essentially same method as described in example 10. Thus, the primer extension method and digestion with Eco RI to obtain 0.6 kbp DNA fragment (Fragment 4, depicted in Fig. 7).

The protein-coding sequence of lignin peroxidase was prepared by the primer extension method and digestion with Hin dIII to obtain 1.7 kbp DNA fragment (Fragment 5, depicted in Fig. 6).

The aforementioned two fragments 4 and 5 were then ligated with T4DNA ligase to the E. coli. vector pUC19 doubly digested with Eco RI and Hin dIII to obtain the plasmid pRPgHL1 containing both fragments (depicted in Fig. 7).

Example 13: Construction expression vector of lignin peroxidase (6)

The plasmid pUCR1 containing the OCTase gene was digested with Eco RI and the cohesive site was treated with Klenow Fragment to give blunt ends. Then Hin dIII linker (8 mer) was ligated to the plasmid to make a Hin dIII site. In addition, this plasmid was digested with Sal I and the cohesive site was treated with Klenow Fragment to give blunt ends followed by the self-ligation to remove Sal I site.

The aforementioned reconbinant DNA was then digested with Hin dIII to obtain the 4.2 kbp DNA fragment containing the OCTase gene (depicted in Fig. 8). This fragment was inserted into the Hin dIII site of the plasmid prepared in Example 8 to obtain the reconbinant DNA pRPC1 (depicted in Fig. 9). The 1.8 kbp DNA fragment containing the protein-coding region of lignin peroxidase (prepared in example 8) was inserted into the Sal I site of the plasmid pRPC1 to obtain the lignin peroxidase expression vector pRPgHLC1 (depicted in Fig. 10).

Example 14: Preparation of C. hirsutus transformed with the lignin peroxidase expression vector

The plasmids, pPgHLC1, pPcHLC1, pPSgHL1, pPSproHL1, and pRPgHL1 (prepared in example 8, 9, 10, 11, and 12, respectively) were co-transformed with the plasmid pUCR1 containing the OCTase gene of C. hirsutus as the marker to the Arg$^-$ auxotrophic mutant OJI-1078 of C. hirsutus, using the PEG method or the electroporation method, to give the transformants pPgHLC1/OJI-1078, pPcHLC1/OJI-1078, pPSgHL1/OJI-1078, pPSproHL1/OJI-1078, and pRPgHL1/OJI-1078, respectively. The plasmid pRPgHLC1 (prepared in example 13) was transformed to the aforementioned OJI-1078, using the PEG method or the electroporation method, to give the transformants pRPgHLC1/OJI-1078. The aforementioned transformations were successful in spite of the form of the plasmids, such as the circular or the linear. The transformation condition was as described below.

To 100 μl of protoplast suspension (about 10^7 plotoplasts/100 μl was added 2 μg of plasmid (circular or linear form) as well as 0.2 μg of plasmid pUCR1 as the selection marker. The mixture was kept on ice for 30

min. In the case of plasmid pRPgHLC1 (prepared in example 13), only 2 μg of plasmid pRPgHLC1 was added to the protoplasts and kept on ice for 30 min. To the resultant mixture was added the same volume of PEG solution (50% PEG3400, 20 mM pH 6.4 MOPS) and kept on ice for 30 min. The resultant protoplast solution was filled to 10 ml with MM containing 0.5M sucrose, 150 μg/ml leucine and 1% agar, poured over plates, and incubated at 28°C for four days to obtain the transformants.

The transformation of the plasmid containing lignin peroxidase was confirmed by the Southern hybridization of the genomic DNA of the transformant.

Example 15: Culture of the transformant and activity of the lignin peroxidase

The five transformants, pPgHLC1/OJI-1078, pPcHLC1/OJI-1078, pPSgHL1/OJI-1078, pPSproHL1/OJI-1078, and pRPgHL1/OJI-1078, prepared in example 14, were shaken-cultured in 100 ml of a GP medium (described in example 1) at 28°C for 7 days. The resultant culture broth was centrifuged to give the supernatant.

On the other hand, the transformants pRPgHLC1/OJI-1078 was cultured in 100ml of the synthetic medium described in Example 1 at 28°C for 10 days. The resultant culture broth was centrifuged to give the culture supernatant.

The activity of lignin peroxidase was measured as described below. To a solution consisting of 50 μl of 8mM veratryl alcohol and 200 μl of 0.5M, pH3, sodium tartrate buffer, was added 700 μl of the culture supernatant and 50 μl of 5.4 mM hydrogenperoxide, successively. The activity (1 unit: production of 1 μ mol /L min) of lignin peroxidase was measured for the increase of the absorption at 310 nm resulting from the oxidation of veratryl alcohol to veratraldehyde. The activities of the lignin peroxidase of the aforementioned culture supernatant of the transformants were in a range of 20 - 100 units/ml. Whereas, in the case of the control experiment culturing C. hirsutus OJI-1078 without the transformation of the plasmid, no lignin peroxidase activity was observed.

Example 16: Construction of phenoloxidase expression vector

The genomic OCTase gene of C. hirsutus contains a promoter region at the 2 kbp upstream of the initiation codon ATG. Therefore, this promoter can be connected to the phenoloxidase gene consisting of the signal-peptide-coding sequence and the protein-coding sequence for the secretional production of phenoloxidase of C. hirsutus OJI-1078.

In detail, the 5.5 kbp Sal I fragment of the OCTase gene was ligated with T4DNA ligase to the Sal I site of phage vector M13mp19, then the complementary DNA was synthesized using the primer extension method and the resultant DNA was digested with Eco RI to obtain 0.6 kbp DNA fragment (Fragment 4, depicted in Example 10).

On the other hand, a plasmid containing the genomic phenoloxidase was digested with Eco RI to obtain the 4.2 kbp DNA fragment of the protein-coding region of the phenoloxidase (fragment 6).

The aforementioned two fragments 4 and 6 were then ligated with T4DNA ligase to the E. coli vector pUC18 digested with Eco RI to obtain the plasmid pRPgPO1 containing both fragments (depicted in Fig. 11).

Example 17: Preparation of the transformed C. hirsutus using the phenoloxidase expression vector

The aforementioned plasmid pRPPCM1 was transformed to C. hirsutus OJI-1078 by the method as described in example 14 to obtain the transformant pRcPO1/OJI-1078.

In addition, the genomic phenoloxidase gene pPO1 (Japanese Patent Application, FERM BP-2793) was also transformed to C. hirsutus OJI-1078 to obtain the transformant pPO1/OJI-1078 for the purpose of the gene dosage effect.

Example 18: Secretional production of phenoloxidase and activity of phenoloxidase

The transformants pRcPO1/OJI-1078 prepared in Example 17 was shaken-cultured at 28°C for 20 days in 100 ml of the synthetic medium described in Example 1. The resultant culture broth was centrifuged to give the culture supernatant.

Similarly, the transformants pPO1/OJI-1078 was cultured in the aforementioned GP medium to the culture supernatant.

The phenoloxidase activities of the both culture supernatants were 20-50% higher than those of the control experiments using C. hirsutus OJI-1078 without the transformation of the plasmids.

Example 19: Construction of expression plasmid of luciferase

A commercially available enhancer vector (Pica Gene, produced by Toyo Ink Co. Ltd., Japan), containing luciferase gene was digested with restriction endonucleases BamHI and BglII to obtain the 3 kbp DNA fragment of the luciferase structural gene. The fragment was then subcloned at the BamHI site of plasmid pPC1 prepared in Example 8, to obtain the luciferase expression plasmid designated as pPLUCC1(depicted in Fig. 12).

Example 20: Preparation of transformed C. hirsutus OJI-1078 with pPLUCC1 and luciferase activity

C. hirsutus OJI-1078 was co-transformed with the plasmids pPLUCC1 and pUCR1. The transformant was cultured in 100 ml of GP medium described in the experiment 1, and was shaken-cultured at 28°C for 7 days.

The cells were harvested by centrifugation, washed with sterilized water, and frozen with liquefied nitrogen. The cell bodies were ground with a mortar. The ground cell was transferred to 30 ml centrifugation tube, suspended in a cell extraction buffer (25mM Tris-phosphate, pH 7.8, 2mM DTT, 2mM 2-diaminocyclohexane-N,N,N',N'-tetraacetic acids, 10% glycerol, 1% Triton X-100), and then left at room temperature for 10 to 15 min. The resultant mixture was centrifuged to remove mycelial debris. Finally, to 20 µl of the supernatant was added the 100 µl of a luciferin solution (attached in the kit) to give luminescence in a darkroom indicating the expression of the luciferase.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: OJI PAPER CO.LTD
(B) STREET: 7-5 Ginza 4-chome, Chuo Ku
(C) CITY: TOKYO 104
(E) COUNTRY: JAPAN
(F) POSTAL CODE (ZIP): none

(ii) TITLE OF INVENTION: ORNITHINE CARBAMOYL TRANSFERASE GENE AND UTILIZATION OF THE DNA

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: EP 93301707.8
(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: JP 4-50513
(B) FILING DATE: 09-MAR-1992

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: JP 4-104549
(B) FILING DATE: 23-APR-1992

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 375 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Coriolus hirsutus
(B) STRAIN: IFO 4917

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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Arg Thr Ala Gln Pro Trp Leu Ala Pro Gln Gly Arg Ala Gly Ser Gly
        35                  40                  45
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Leu	Phe	Ser	Lys	Ser	Ile	Ala	Leu	Leu	Phe	Ser	Lys	Arg	Ser	Thr	Arg
65					70					75					80
Thr	Arg	Leu	Ser	Ala	Glu	Thr	Ala	Ala	Leu	Leu	Leu	Gly	Gly	Gln	Ala
				85					90					95	
Leu	Phe	Leu	Gly	Arg	Glu	Asp	Ile	Gln	Leu	Gly	Val	Asn	Glu	Thr	Val
			100					105					110		
Pro	Asp	Ser	Ala	Arg	Val	Ile	Gly	Gly	Met	Cys	Gln	Gly	Ile	Phe	Ala
		115					120					125			
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Val	Pro	Val	Leu	Asn	Ala	Leu	Ser	Ser	Leu	Trp	His	Pro	Thr	Gln	Val
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Leu	Ala	Asp	Ile	Leu	Thr	Leu	His	Glu	His	Ala	Ala	Leu	Phe	Asp	Pro
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Ala	Ser	Ala	Ser	Pro	Thr	Pro	Ser	Ala	Ala	Asp	Ala	Phe	Ser	Gln	Lys
			180					185					190		
Tyr	Thr	Lys	Leu	Gly	Glu	Val	Gly	Pro	Leu	Thr	Val	Ala	Tyr	Val	Gly
		195					200					205			
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Pro	Lys	Ala	Val	Trp	Asp	Arg	Val	Val	Glu	Leu	Gly	Cys	Asp	Lys	Asn
				245					250					255	
Ile	Phe	Trp	Thr	Ala	Asp	Pro	Arg	Ala	Ala	Val	Lys	Gly	Ala	Asp	Leu
			260					265					270		
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		275					280					285			
Gln	Arg	Leu	Lys	Asp	Phe	Ala	Gly	Tyr	Gln	Val	Thr	Glu	Ala	Leu	Cys
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305					310					315					320
Arg	Lys	Gln	Asp	Glu	Val	Asp	Asp	Glu	Val	Phe	Tyr	Gly	Pro	Arg	Ser
				325					330					335	
Leu	Val	Phe	Gln	Glu	Ser	Asp	Asn	Arg	Lys	Trp	Thr	Ile	Met	Ala	Leu
			340					345					350		

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Phe Asp Leu Leu Phe Gly Lys Trp Ser Leu Leu Ala Arg Asn Gly Glu
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Gly Ala Asp Ala Gly Ser Glu
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 375 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Coriolus hirsutus
(B) STRAIN: IFO 4917

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Gly Lys Tyr Ser Asn Ala Pro His Lys Leu Arg Met Pro Ser Gln Ser
    50                  55                  60

Leu Phe Ser Lys Ser Ile Ala Leu Leu Phe Ser Lys Arg Ser Thr Arg
65                  70                  75                  80

Thr Arg Leu Ser Ala Glu Thr Ala Ala Leu Leu Leu Gly Gly Gln Ala
                85                  90                  95

Leu Phe Leu Gly Arg Glu Asp Ile Gln Leu Gly Val Asn Glu Thr Val
            100                 105                 110

Pro Asp Ser Ala Arg Val Ile Gly Gly Met Cys Gln Gly Ile Phe Ala
        115                 120                 125

Arg Val Gly Asp His Ser Glu Ile Glu Glu Leu Ala Arg Tyr Ser Pro
    130                 135                 140

Val Pro Val Leu Asn Ala Leu Ser Ser Leu Trp His Pro Thr Gln Val
145                 150                 155                 160

Leu Ala Asp Ile Leu Thr Leu His Glu His Ala Ala Leu Phe Asp Pro
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Ala Ser Ala Ser Pro Thr Pro Ser Ala Ala Asp Ala Phe Ser Gln Lys
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Gly His Gln Leu Ala Val Ala Ser Pro Glu Asn Asp Arg Tyr Arg Ala
225                 230                 235                 240

Pro Lys Ala Val Trp Asp Arg Val Val Glu Leu Gly Cys Asp Lys Asn
                245                 250                 255

Ile Phe Trp Thr Ala Asp Pro Arg Ala Ala Val Lys Gly Ala Asp Leu
            260                 265                 270

Val Val Thr Asp Thr Trp Ile Ser Met Gly Gln Glu Ala Glu Lys Ala
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Gln Arg Leu Lys Asp Phe Ala Gly Tyr Gln Val Thr Glu Ala Leu Cys
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Arg Glu Gly Gly Ala Asn Pro Asp Trp Lys Phe Met His Cys Leu Pro
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Arg Lys Gln Asp Glu Val Asp Asp Glu Val Phe Tyr Gly Pro Arg Ser
                325                 330                 335

Leu Val Phe Gln Glu Ser Asp Asn Arg Lys Trp Thr Ile Met Ala Leu
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Gly Ala Asp Ala Gly Ser Glu
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1280 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Coriolus hirsutus
(B) STRAIN: IFO 4917

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 78..1202

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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CCCAGCCCTG GCTCGCGCCC CAGGGGCGCG CAGGCAGCGG CGGCAAGTAC AGCAACGCCC    240
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2540 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Coriolus hirsutus
(B) STRAIN: IFO 4917

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 653..2340

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1064..1116

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 1539..1928

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2083..2140

(ix) FEATURE:
(A) NAME/KEY: intron
(B) LOCATION: 2215..2276

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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CATCATCACG CACTCCTACC ACCTCAAGCG CACCGCCCAG CCCTGGCTCG CGCCCCAGGG     780
GCGCGCAGGC AGCGGCGGCA AGTACAGCAA CGCCCCGCAC AAGCTGCGCA TGCCGTCGCA     840
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CTCCGCCGAG ACCGCCGCCC TCCTCCTCGG CGGGCAGGCG CTCTTCCTCG GGCGGGAGGA     960
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TGCCGTCCAC GCTCGTCACG CTCACCCATC CCCCAGGAAC TCGCCCGGTA CTCGCCCGTG 1140

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ACGCTGCACG AGCACGCCGC GCTCTTCGAC CCGGCCTCCG CGAGCCCCAC GCCCTCCGCC 1260

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CCTCTTCCGC ATCTGTTACC CTGGCGATCC ACGAACGTGT ACACAGAATA CTGACAGCTG 1920

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GCTGGTGTTC CAGGAGTCGG ACAACCGGAA GTGGACGATC ATGGCGTTGT TCGAGTGCGT 2220

TCTCGCGTTA GTTGTGCTGA TACGCCCGAT GTAGGAGTGC TGATCACGTT CTGCAGCCTT 2280

CTCTTTGGAA AGTGGTCGCT GCTCGCGCGG AACGGGGAGG GCGCAGATGC GGGGTCGGAG 2340

TAGACACAGA TAGCGTAGAC GGAGTAATGC TGGCACAACG ATCGTCTGTC CGTGCGGGGT 2400

ATGTTGCGCG TGAATGTTCC TGGAGACTTG CCTGGAATGG ACAAGATTGG CCACGTGCCG 2460

CGCATGACGT CTGCCAGTCC GGGGTAGCGC CCAGCCCGGC CGAGCTCTGT TCTCCTCCTC 2520

CTCCCCTTCT TCCCTCTCTT 2540

Claims

1. An isolated structural gene coding for ornithine carbamoyl transferase (OCTase) of Coriolus hirsutus.

2. An isolated structural gene according to claim 1 wherein the gene encodes the amino acid sequence shown in SEQ ID NO: 1, or SEQ ID NO: 2.

3. An isolated OCTase gene comprising a structural gene coding for OCTase and a control region controlling the expression of the structural gene.

4. An isolated OCTase gene according to claim 3, wherein the OCTase gene essentially consists of the nucleotide sequence shown in SEQ ID NO: 4.

5. A recombinant linear or circular DNA comprising a structural gene coding for OCTase of C. hirsutus, or an OCTase gene comprising a structural gene coding for OCTase of C. hirsutus and a control sequence controlling the expression of the OCTase.

6. A recombinant linear or circular DNA according to claim 5, wherein the structural gene encodes the amino acid sequence shown in SEQ ID NO: 1 or SEQ ID NO: 2.

7. A recombinant linear or circular DNA according to claim 6, wherein the OCTase gene consists essentially of the nucleotide sequence shown in SEQ ID NO: 4.

8. A recombinant circular DNA according to claim 6, wherein the circular DNA is a plasmid.

9. A recombinant linear or circular DNA comprising:
   (1) a structural gene coding for OCTase of C. hirsutus, or an OCTase gene comprising a structural gene coding for OCTase of C. hirsutus and a control sequence for controlling the expression of the structural gene;
   (2) an expression control sequence operable in a basidiomycete of the genus Coriolus; and
   (3) a gene coding for a desired protein under the control by the expression control sequence (2).

10. A recombinant linear or circular DNA according to claim 9, wherein the expression control sequence is a promoter selected from the group consisting of a promoter of phenoloxidase gene, a promoter of lignin peroxidase gene and a promoter of OCTase gene.

11. A recombinant linear or circular DNA according to claim 9, wherein the recombatant linear or circular DNA further comprises a DNA sequence coding for signal peptide present upstream of the gene coding for the desired protein and linked in frame with the gene coding for the desired protein.

12. A recombinant linear or circular DNA according to claim 11 wherein the signal peptide is selected from the group consisting of a signal peptide of phenoloxidase and a signal peptide of lignin peroxidase.

13. An auxotrophic mutant of a basidiomycete of the genus Coriolus deficient in an ability to express OCTase.

14. A host-vector system comprising:
   (1) an OCTase gene comprising a strucutural gene coding for OCTase of C. hirsutus and a control sequence for controlling the expression of the structural gene: and
   (2) an auxotrophic mutant of a basidiomycete of the genus Coriolus deficient in an ability to express OCTase.

15. A host-vector system for expression of a desired protein according to claim 14, further comprising a gene coding for the desired protein and a control sequence for expression of the gene coding for the desired protein.

16. A process for production of a transformant basidiomycete of the genus Coriolus capable of expressing a desired protein, comprising the step of:
   (a) cotransforming an auxotrophic mutant of a basidiomycete of the genus Coriolus deficient in an ability to express OCTase, with a linear or circular DNA comprising a structural gene coding for OCTase of C. hirsutus, or an OCTase gene comprising a structural gene coding for OCTase of C. hirsutus and a

control sequence controlling the expression of the OCTase, and with a linear or circular DNA comprising a gene coding for a desired protein and a control sequence for controlling the expressions of the gene coding for the desired protein; and
(b) selecting a transformant capable of growing in a medium-lacking arginine.

17. A process for production of a transformant basidiomycete of the genus Coriolus capable of expressing a desired protein, comprising the steps of:
(a) transforming an auxotrophic mutant of a basidiomycete of the genus Coriolus deficient in an ability to express OCTase, with a linear or circular DNA comprising:
(1) a structural gene coding for OCTase of C. hirsutus, or an OCTase gene comprising a structural gene coding for OCTase of C. hirsutus and a control sequence for controlling the expression of the structural gene;
(2) an expression control sequence operable in a basidiomycete of the genus coriolus; and
(3) a gene coding for a desired protein under the control by the expression control sequence (2), and.
(b) selecting a transformant capable of growing in a medium lacking arginine.

18. A transformant basidiomycete obtainable by a process according to claim 16, capable of producing a desired protein.

19. A transformant basidiomycete obtainable by a process according to claim 17, capable of producing a desired protein.

20. A process for production of a desired protein, comprising the steps of:
culturing a transformant basidiomycete according to claim 18 to produce the desired protein; and recovering the desired protein from the culture.

21. A process for production of a desired protein, comprising the steps of:
culturing a transformant basidiomycete according to claim 19, to produce the desired protein; and recovering the desired protein from the culture.

Fig.1

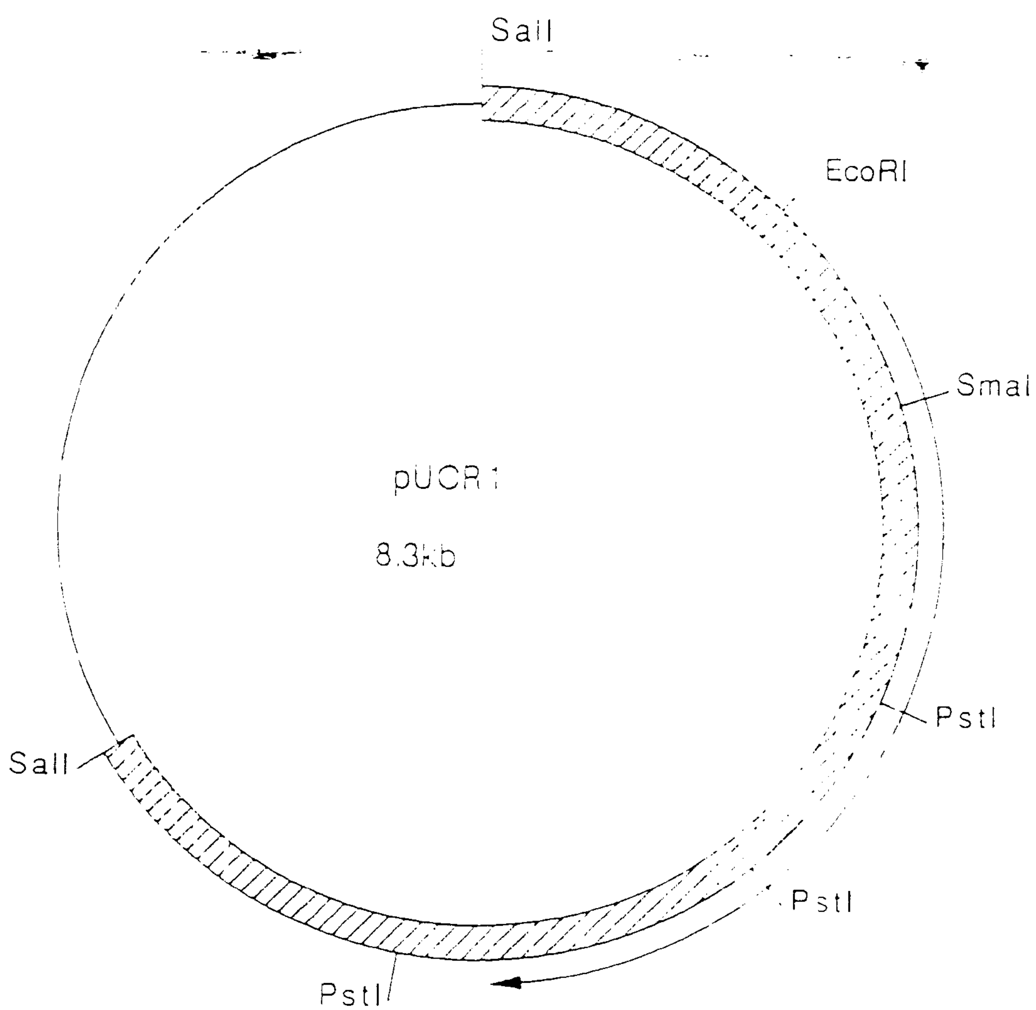
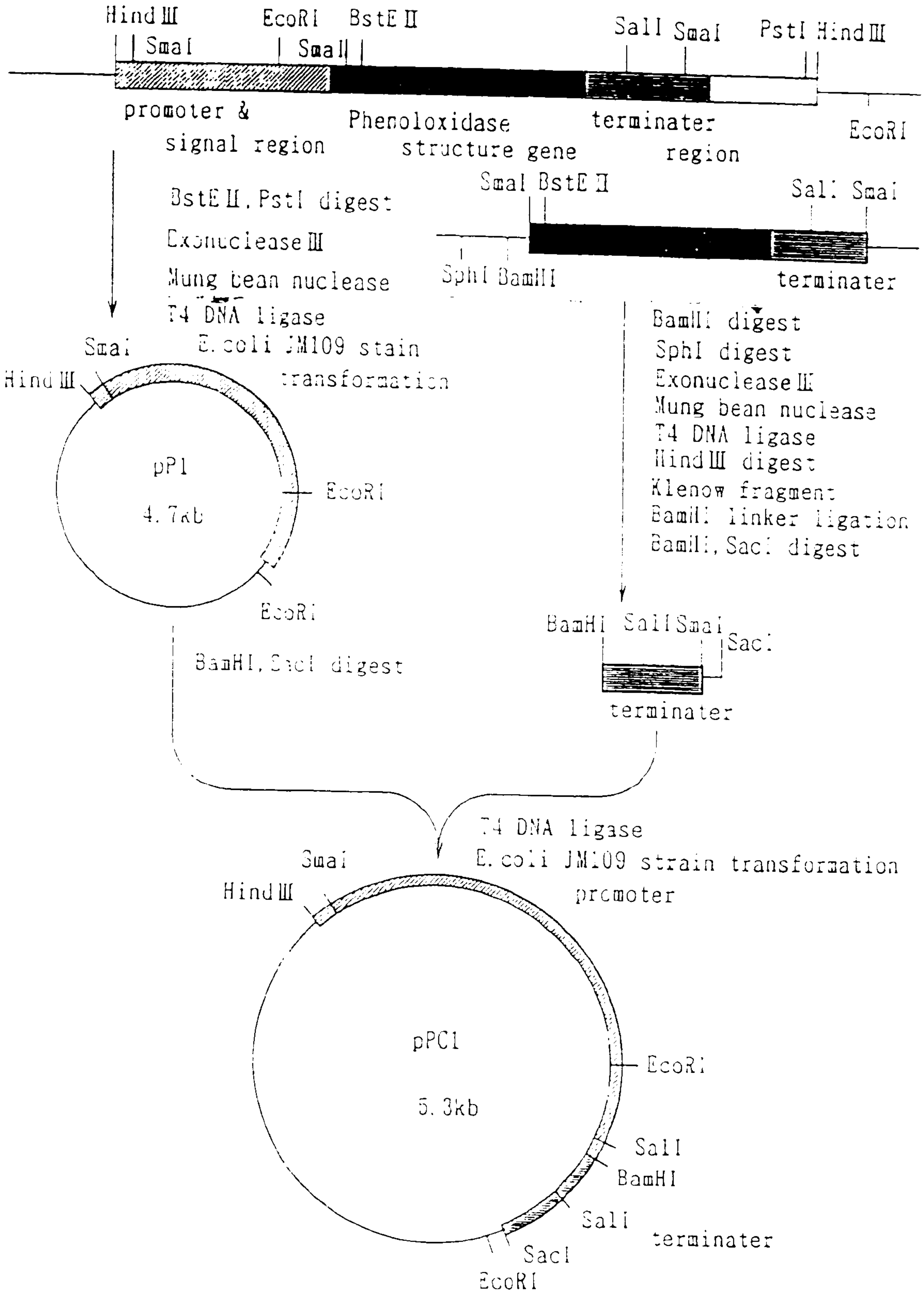


Fig.2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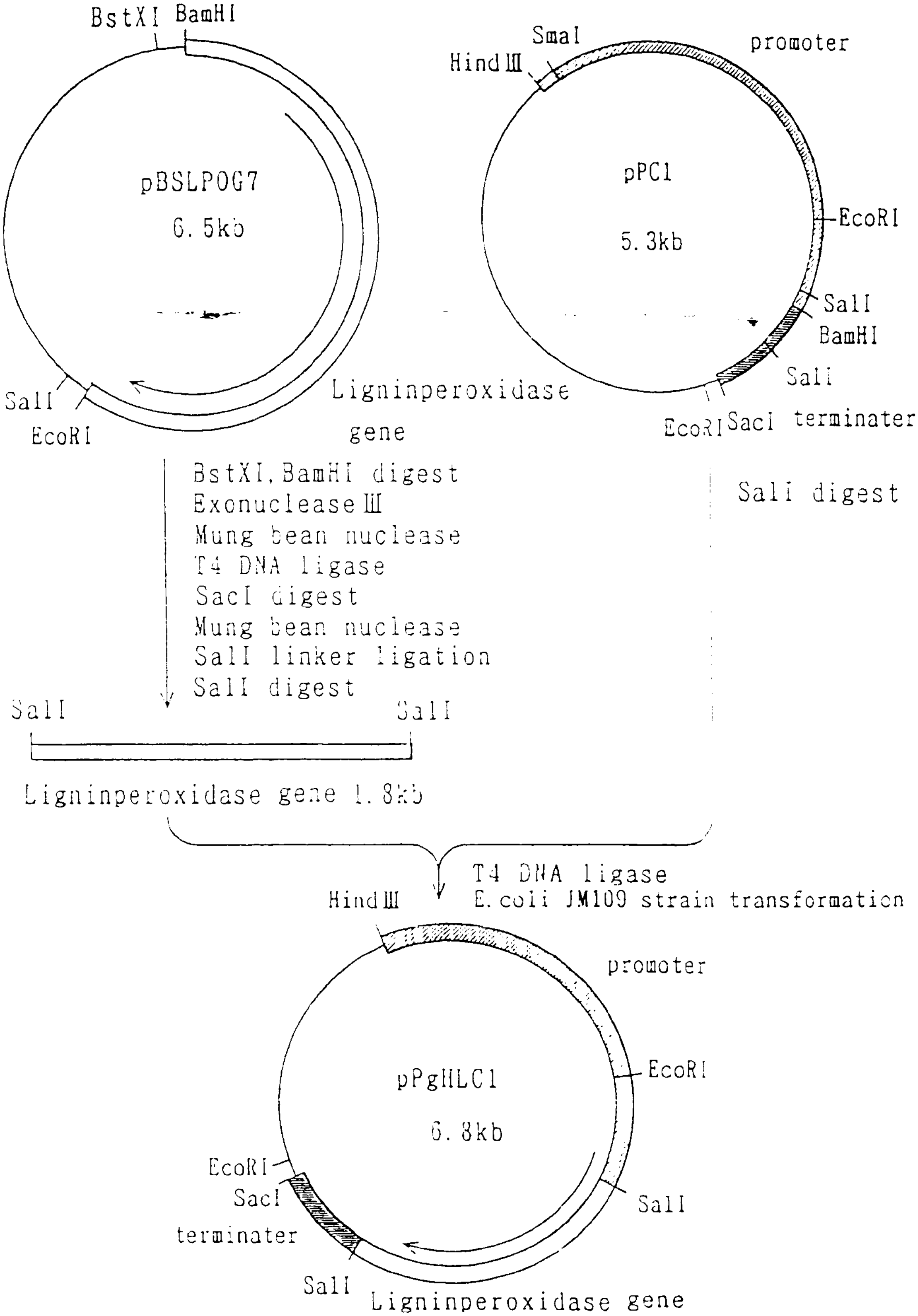
EcoRI
SmaI
BstEII
SalI
SmaI
PstI
HindIII
promoter &
signal region
Phenoloxidase
structure gene
terminater
region
EcoRI
BstEII, PstI digest
Exonuclease III
Mung bean nuclease
T4 DNA ligase
E. coli JM109 strain
transformation
SmaI
BstEII
SalI
SmaI
SphI
BamHI
terminater
BamHI digest
SphI digest
Exonuclease III
Mung bean nuclease
T4 DNA ligase
HindIII digest
Klenow fragment
BamHI linker ligation
BamHI, SacI digest
SmaI
HindIII
pP1
4.7kb
EcoRI
EcoRI
BamHI, SacI digest
BamHI
SalI
SmaI
SacI
terminater
T4 DNA ligase
E. coli JM109 strain transformation
promoter
SmaI
HindIII
pPC1
5.3kb
EcoRI
SalI
BamHI
SalI
terminater
SacI
EcoRI

Fig.3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SalI
EcoRI
Ligninperoxidase gene
HindIII
SmaI
promoter
pPC1
5.3kb
EcoRI
SalI
BamHI
SalI
EcoRI
SacI
terminater
BstXI, BamHI digest
Exonuclease III
Mung bean nuclease
T4 DNA ligase
SacI digest
Mung bean nuclease
SalI linker ligation
SalI digest
SalI digest
SalI
SalI
Ligninperoxidase gene 1.8kb
T4 DNA ligase
E. coli JM109 strain transformation
HindIII
promoter
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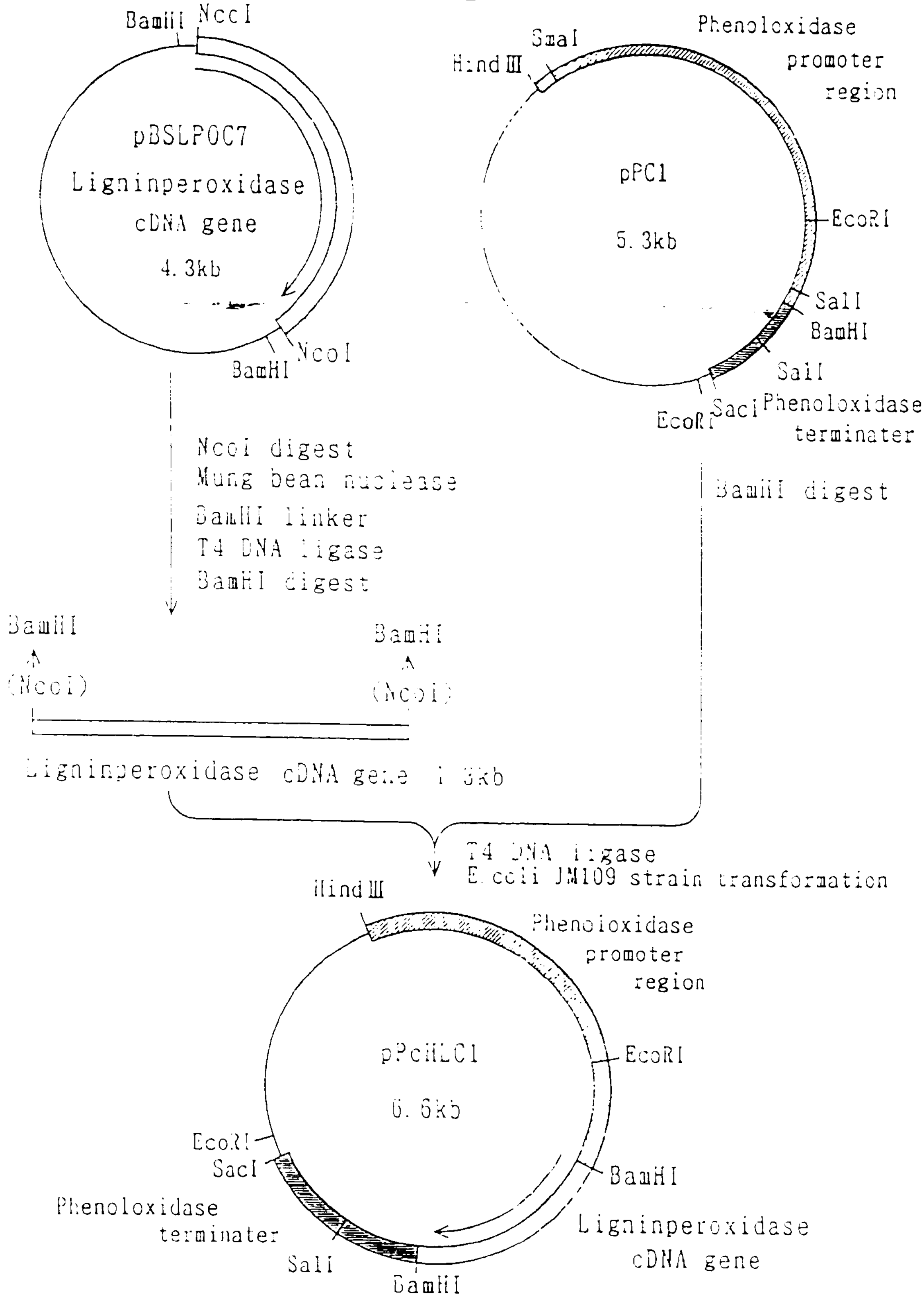
6.8kb
EcoRI
EcoRI
SacI
terminater
SalI
SalI
Ligninperoxidase gene

Fig.4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Ligninperoxidase
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BamHI
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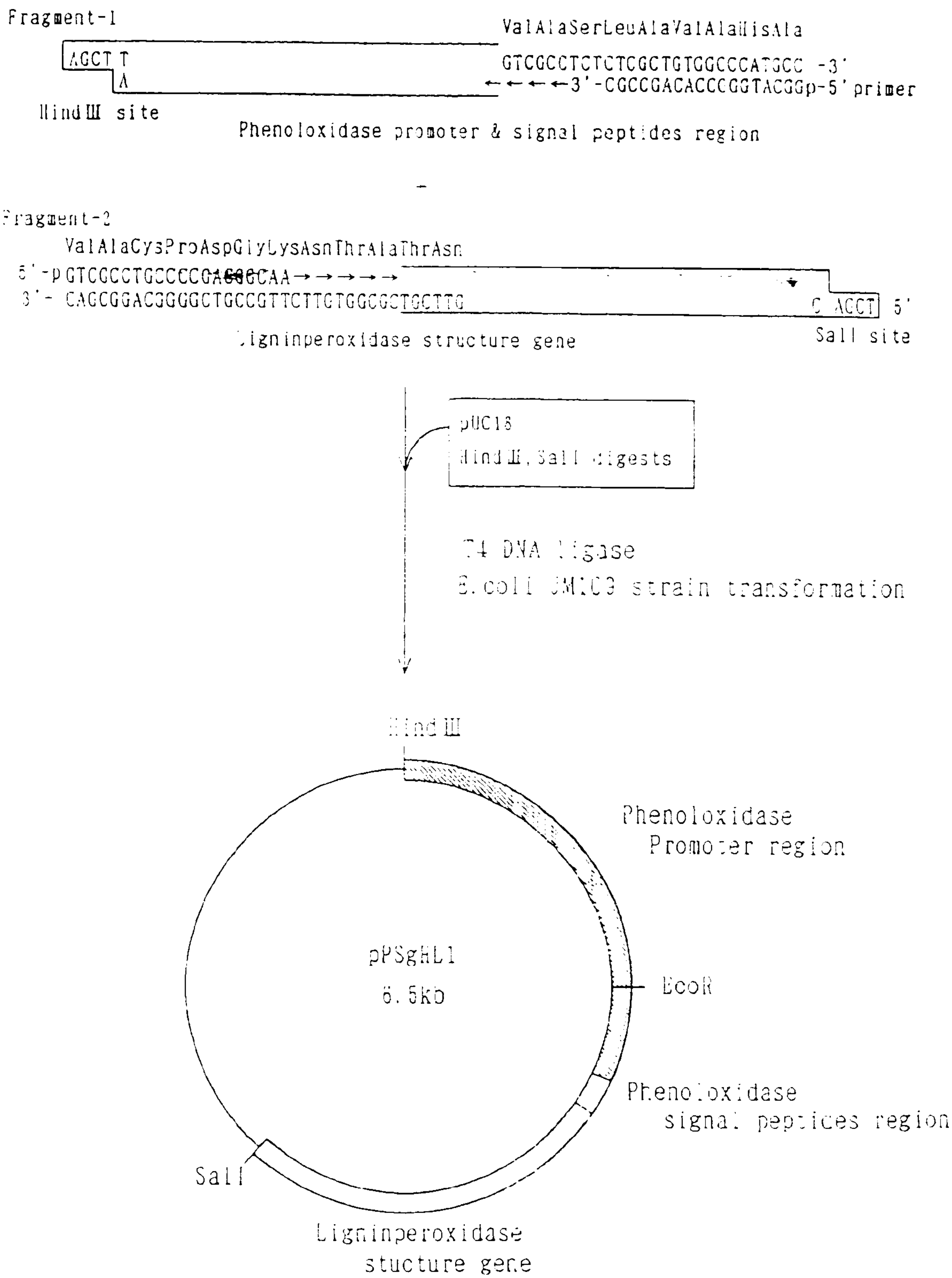
SmaI
HindIII
Phenoloxidase
promoter
region
pPC1
5.3kb
EcoRI
SalI
BamHI
SalI
EcoRI
SacI
Phenoloxidase
terminater
NcoI digest
Mung bean nuclease
BamHI linker
T4 DNA ligase
BamHI digest
BamHI digest
BamHI
(NcoI)
BamHI
(NcoI)
Ligninperoxidase cDNA gene 1.3kb
T4 DNA ligase
E.coli JM109 strain transformation
HindIII
Phenoloxidase
promoter
region
pPcHLC1
6.6kb
EcoRI
EcoRI
SacI
BamHI
Phenoloxidase
terminater
Ligninperoxidase
cDNA gene
SalI
BamHI

Fig.5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ValAlaSerLeuAlaValAlaHisAla
AGCT T
A
GTCGCCTCTCTCGCTGTGGCCCATGCC -3'
3'-CGCCGACACCCGGTACGGp-5' primer
HindIII site
Phenoloxidase promoter & signal peptides region
Fragment-2
ValAlaCysProAspGlyLysAsnThrAlaThrAsn
5'-pGTCGCCTGCCCCGACGGCAA
3'- CAGCGGACGGGGCTGCCGTTCTTGTGGCGCTGCTTG
C AGCT 5'
Ligninperoxidase structure gene
SalI site
pUC18
HindIII, SalI digests
T4 DNA ligase
E.coli JM109 strain transformation
HindIII
Phenoloxidase
Promoter region
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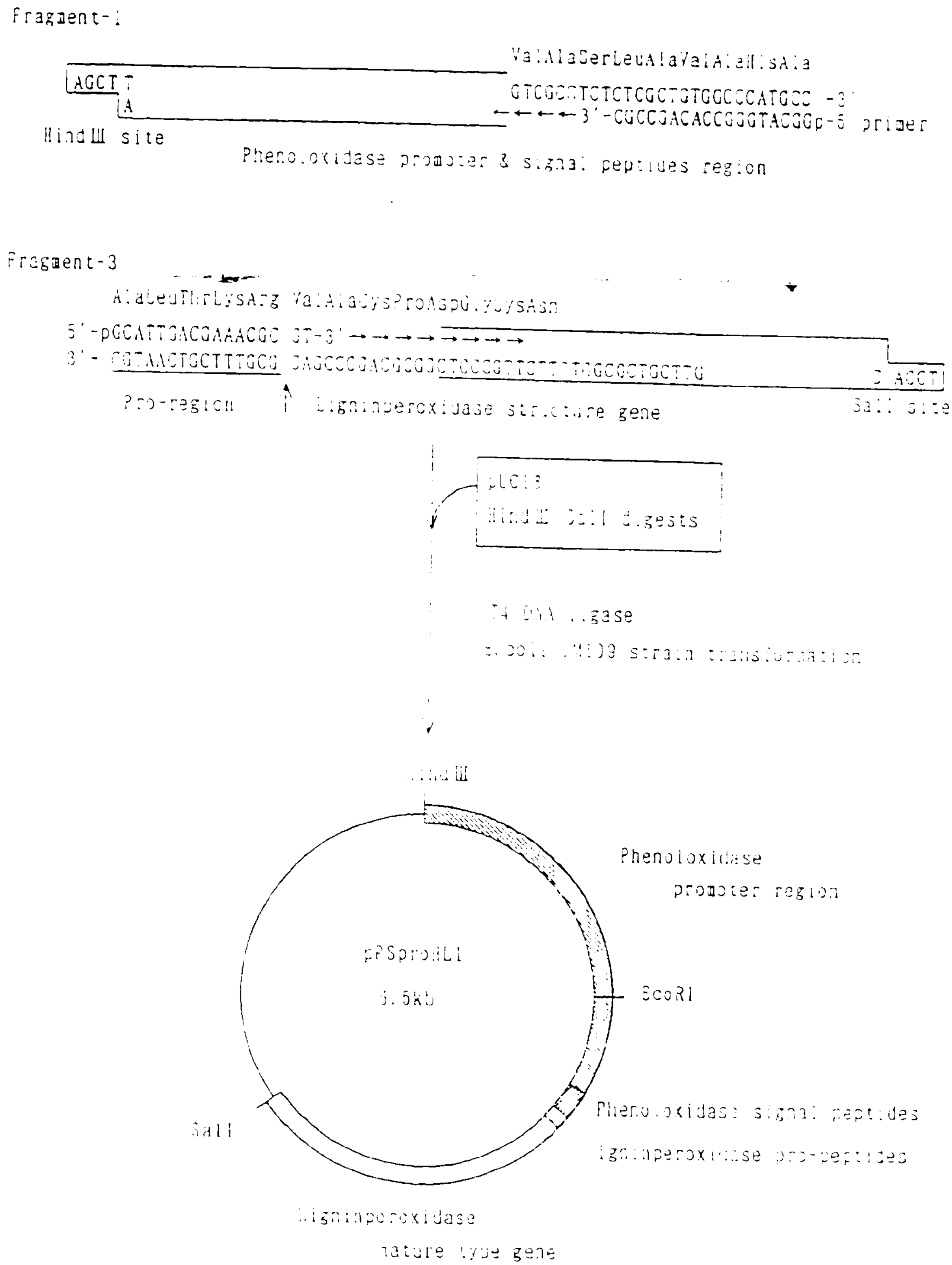
6.5kb
EcoRI
Phenoloxidase
signal peptides region
SalI
Ligninperoxidase
stucture gene

Fig.6



Fragment-1
ValAlaSerLeuAlaValAlaHisAla
AGCT T
A
GTCG TCTCTCGCTGTGGCCCATGCC -3'
3'-CGCCGACACCGGGTACGGp-5 primer
HindIII site
Phenoloxidase promoter & signal peptides region
Fragment-3
AlaLeuThrLysArg ValAlaCysProAspGlyLysAsn
5'-pGCATTGACGAAACGC GT-3'
3'- CGTAACTGCTTTGCG CAGCCCGACGCGGCTGCCGTTCTTTAGCGCTGCTTG
C AGCT
Pro-region
Ligninperoxidase structure gene
SalI site
pUC18
HindIII SalI digests
T4 DNA ligase
E.coli JM109 strain transformation
HindIII
Phenoloxidase
promoter region
pPSproHL1
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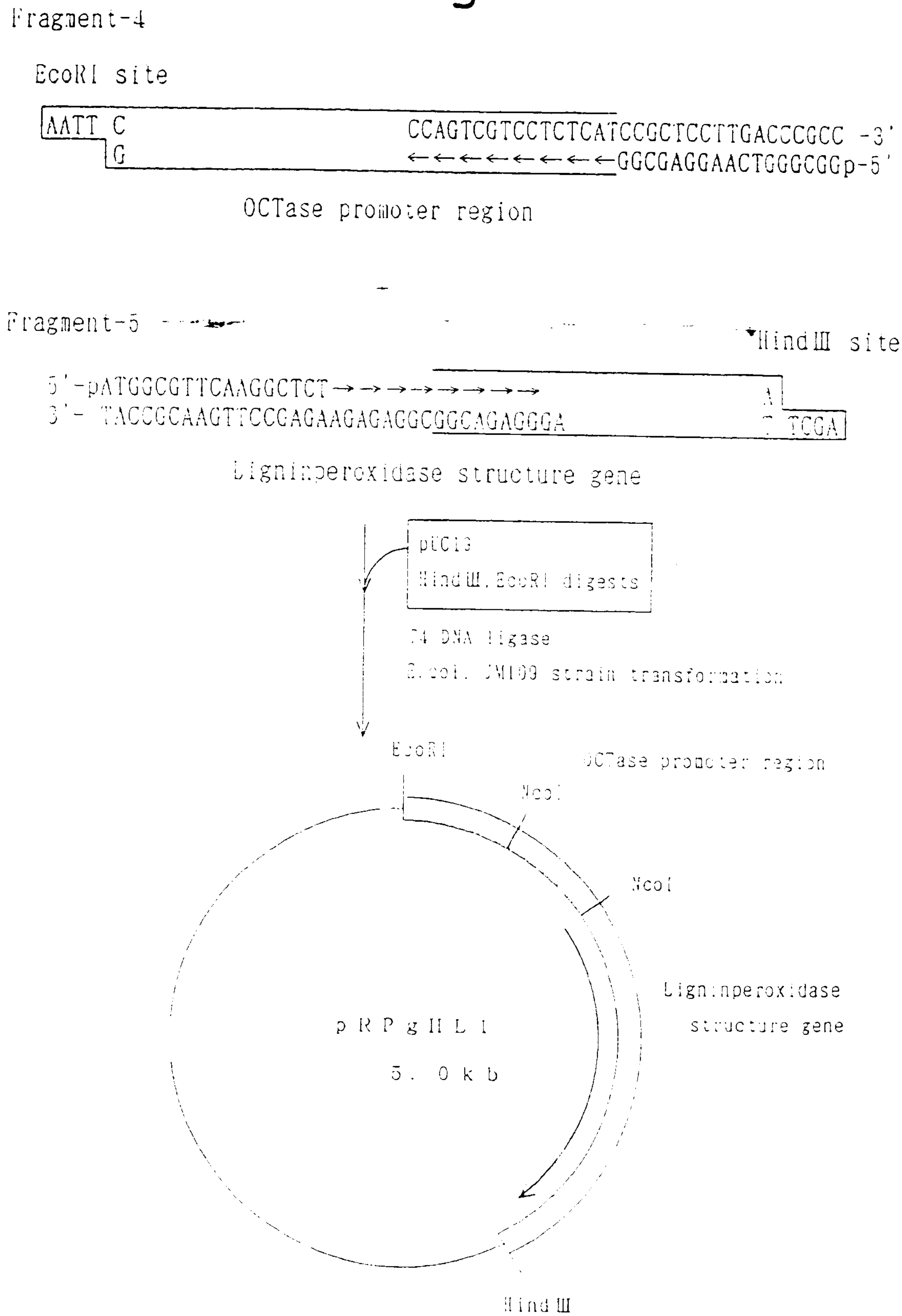
EcoRI
Phenoloxidase signal peptides
Ligninperoxidase pro-peptides
SalI
Ligninperoxidase
mature type gene

Fig.7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EcoRI site
AATT C
G
CCAGTCGTCCTCTCATCCGCTCCTTGACCCGCC -3'
←←←←←←←←GGCGAGGAACTGGGCGGp-5'
OCTase promoter region
Fragment-5
HindIII site
5'-pATGGCGTTCAAGGCTCT→→→→→→→→
3'- TACCGCAAGTTCCGAGAAGAGACGCGGCAGAGGGA
A
T TCGA
Ligninperoxidase structure gene
pUC19
HindIII, EcoRI digests
T4 DNA ligase
E.coli JM109 strain transformation
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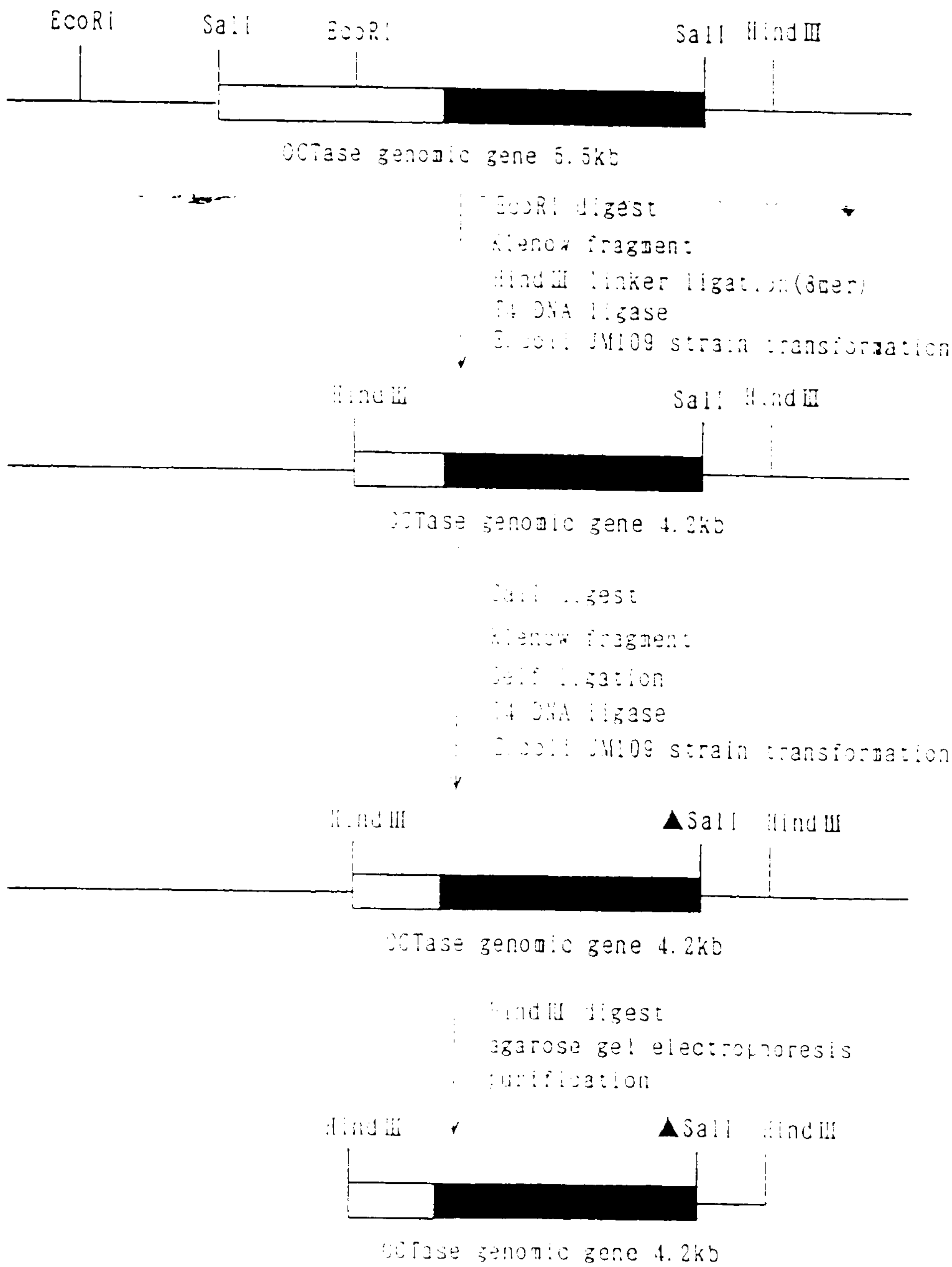
NcoI
NcoI
Ligninperoxidase
structure gene
pRPgHL1
5.0kb
HindIII

Fig.8


EcoRI
SalI
EcoRI
SalI
HindIII
OCTase genomic gene 6.5kb
EcoRI digest
Klenow fragment
HindIII linker ligation(8mer)
T4 DNA ligase
JM109 strain transformation
HindIII
SalI
HindIII
OCTase genomic gene 4.2kb
SalI digest
Klenow fragment
Self ligation
T4 DNA ligase
JM109 strain transformation
HindIII
▲SalI
HindIII
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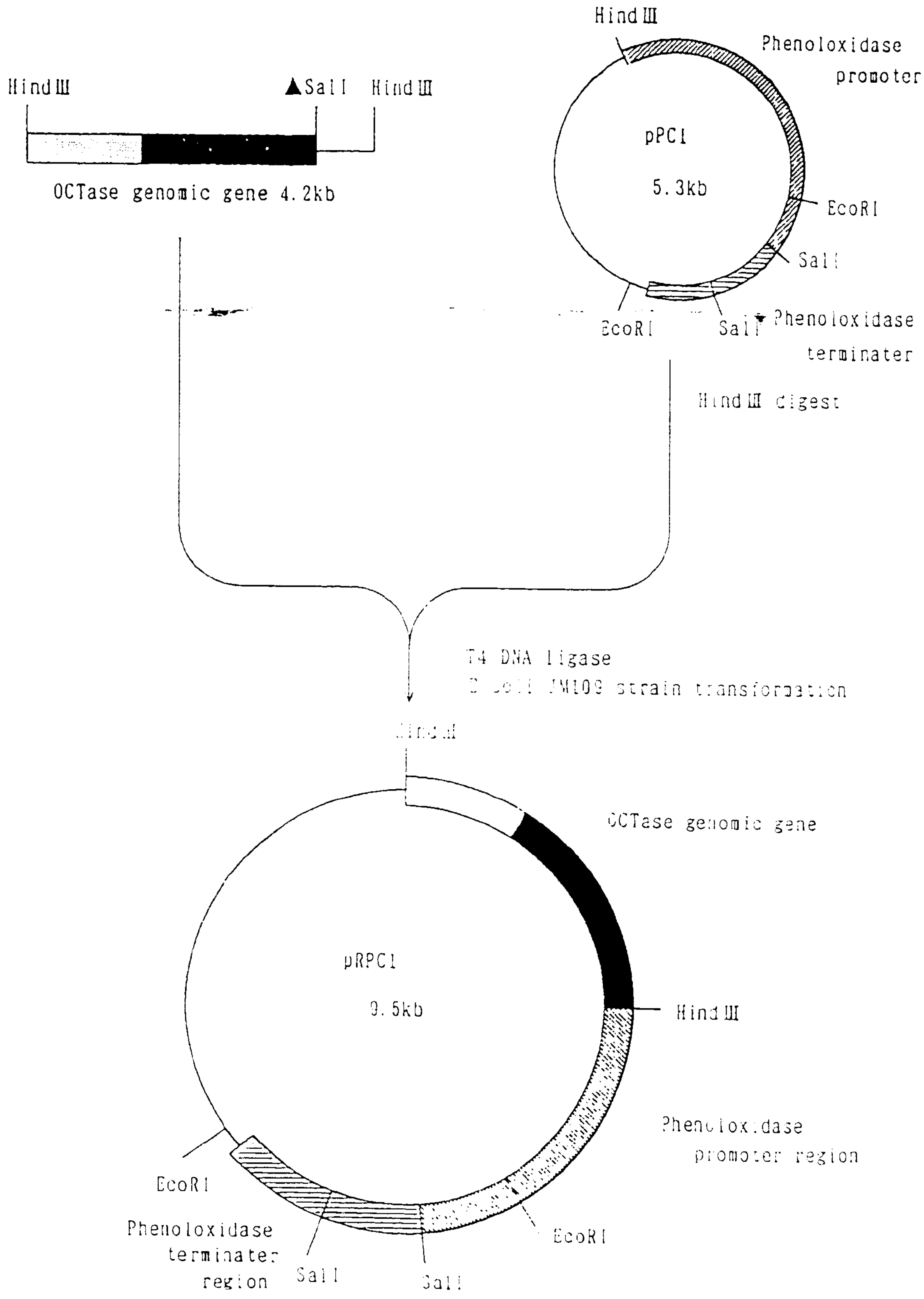
HindIII digest
agarose gel electrophoresis
purification
HindIII
▲SalI
HindIII
OCTase genomic gene 4.2kb

Fig.9


HindIII
Phenoloxidase promoter
HindIII
▲SalI
HindIII
pPC1
5.3kb
OCTase genomic gene 4.2kb
EcoRI
SalI
EcoRI
SalI
Phenoloxidase terminater
HindIII digest
T4 DNA ligase
OCTase genomic gene
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HindIII
Phenoloxidase promoter region
EcoRI
Phenoloxidase terminater region
SalI
SalI
EcoRI

Fig.10

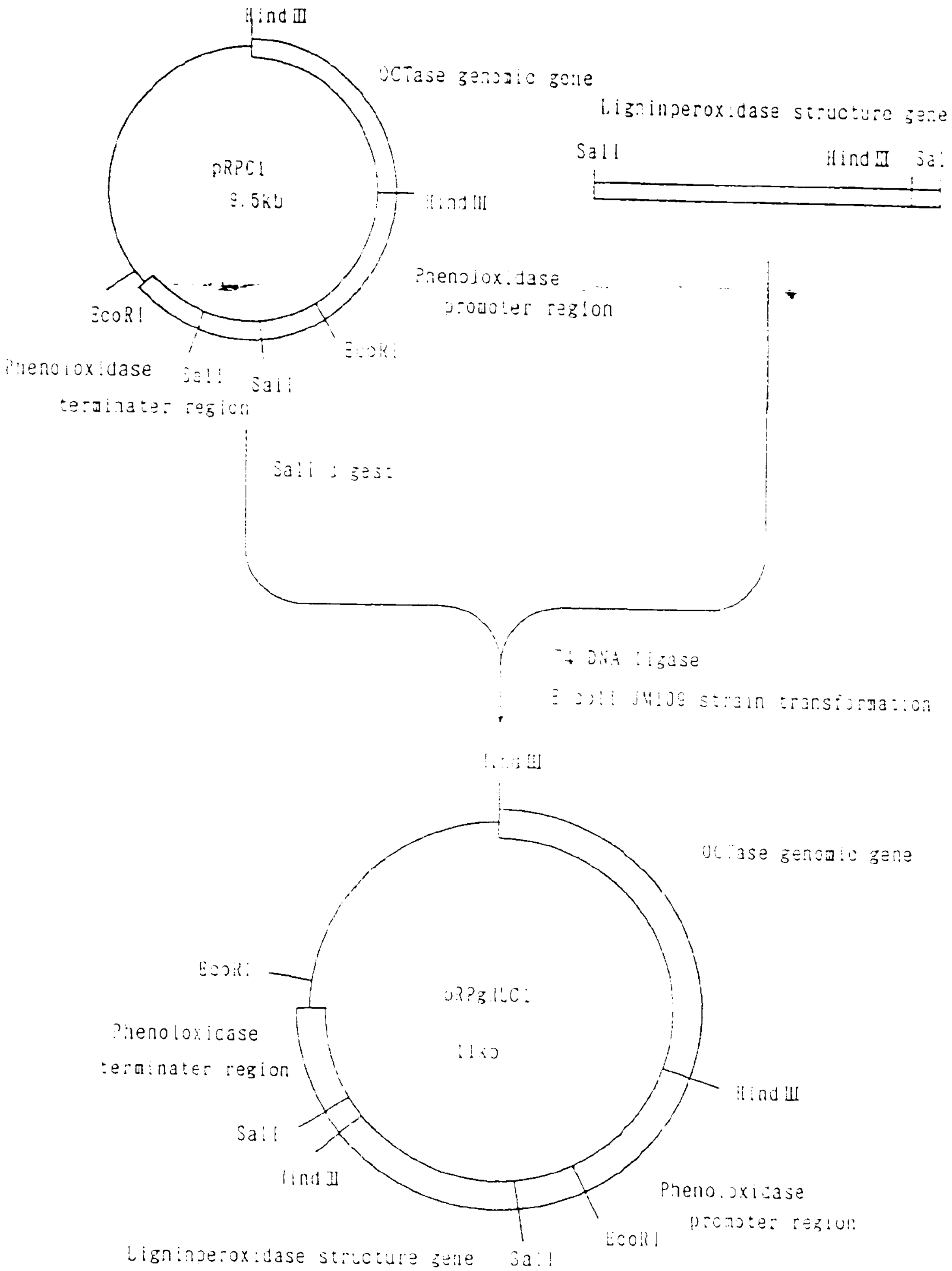

Hind III
OCTase genomic gene
pRPC1
9.5Kb
Hind III
EcoRI
EcoRI
Phenoloxidase
terminater region
SalI
SalI
Ligninperoxidase structure gene
SalI
Hind III
Phenoloxidase
promoter region
SalI digest
T4 DNA ligase
E. coli JM109 strain transformation
OCTase genomic gene
EcoRI
Phenoloxicase
terminater region
Hind III
SalI
Hind III
Phenoloxidase
promoter region
EcoRI
Ligninperoxidase structure gene
SalI

Fig.11

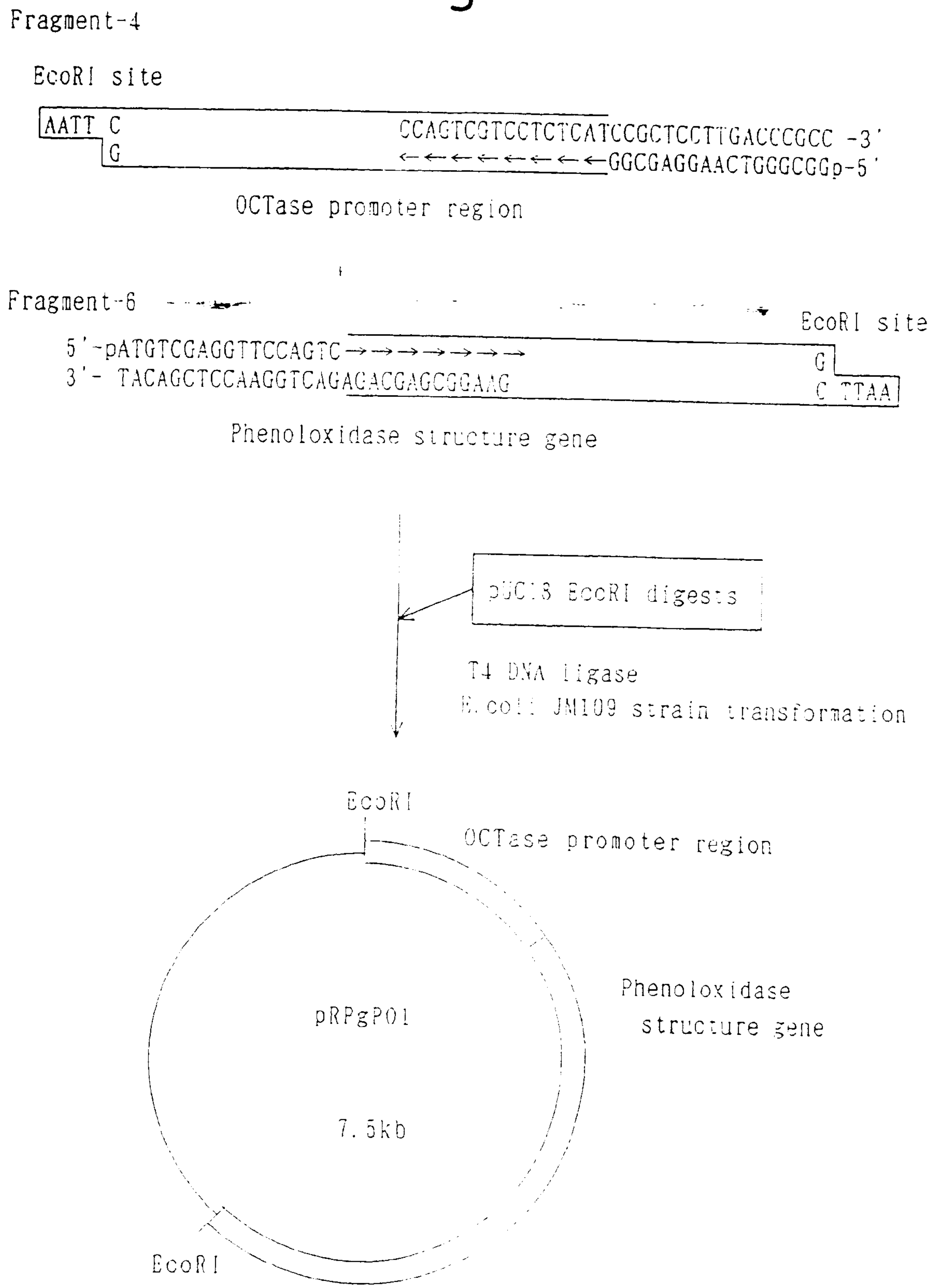
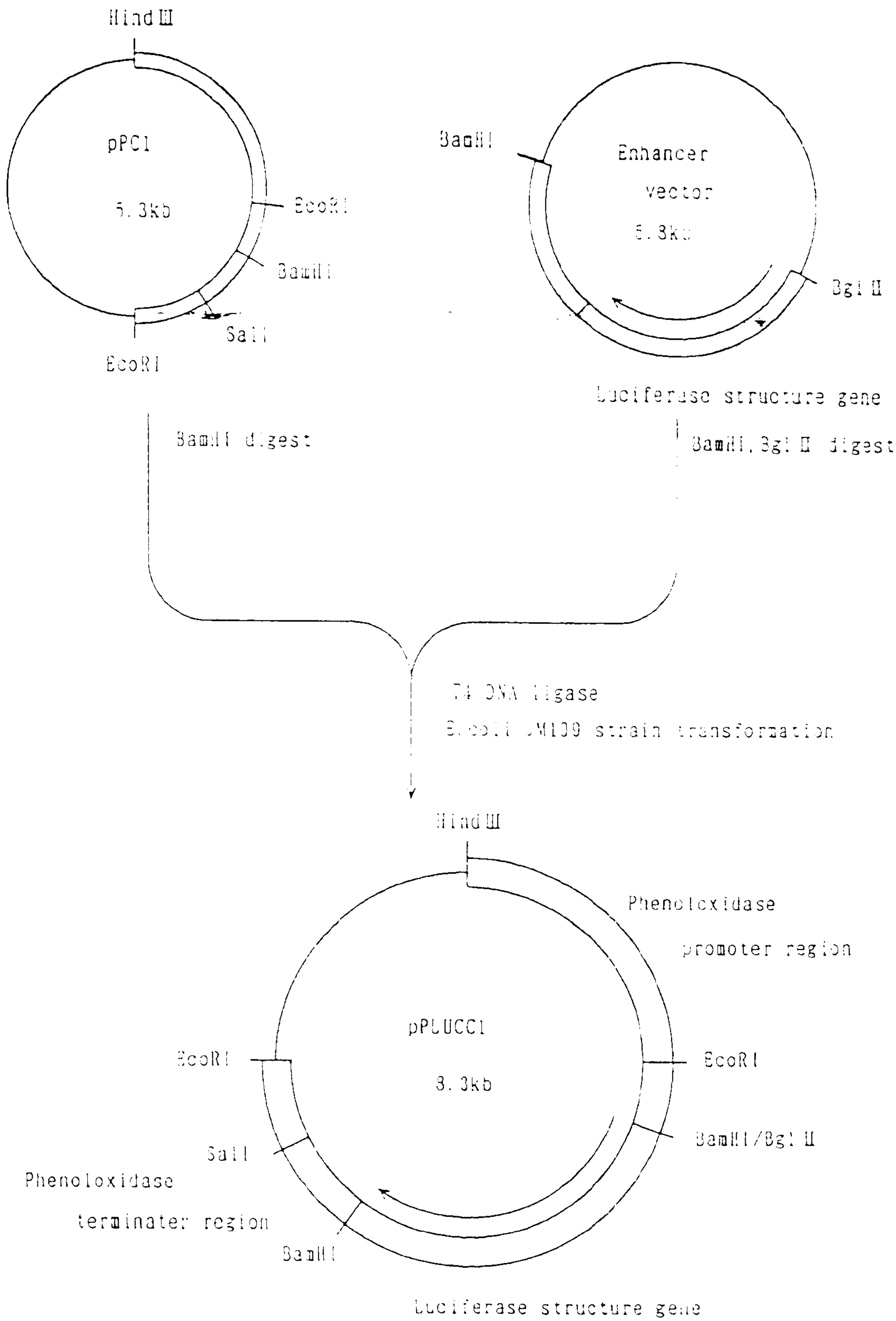

Fragment-4
EcoRI site
AATT C
G
CCAGTCGTCCTCTCATCCGCTCCTTGACCCGCC -3'
GGCGAGGAACTGGGCGGp-5'
OCTase promoter region
Fragment-6
EcoRI site
5'-pATGTCGAGGTTCCAGTC
3'- TACAGCTCCAAGGTCAGAGACGAGCGGAAG
G
C TTAA
Phenoloxidase structure gene
pUC18 EcoRI digests
T4 DNA ligase
E.coli JM109 strain transformation
EcoRI
OCTase promoter region
pRPgP01
7.5kb
Phenoloxidase
structure gene
EcoRI

Fig.12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BamHI
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BamHI
Enhancer vector
5.8kb
BglII
Luciferase structure gene
BamHI digest
BamHI, BglII digest
T4 DNA ligase
E.coli JM109 strain transformation
HindIII
Phenoloxidase promoter region
pPLUCC1
8.3kb
EcoRI
EcoRI
BamHI/BglII
SalI
Phenoloxidase terminater region
BamHI
Luciferase structure gene

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 0 570 096 A3

(12) EUROPEAN PATENT APPLICATION

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(43) Date of publication A2:
18.11.1993 Bulletin 1993/46

(21) Application number: 93301737.8

(22) Date of filing: 08.03.1993

(51) Int Cl.6: C12N 15/54, C12N 15/80, C12N 1/15, C12N 1/14, C12N 15/53, C12N 15/62 // (C12N1/15, C12R1:645)

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23.04.1992 JP 104549/92

(71) Applicant: NEW OJI PAPER CO., LTD.
Tokyo 104 (JP)

(72) Inventors:
- Tsukamoto, Akira
Shinjuku-ku, Tokyo (JP)
- Matsufuji, Mieko
Kawaguchi-shi, Saitama (JP)
- Kita, Yukio
Meguro-ku, Tokyo (JP)

(74) Representative: Arthur, Bryan Edward et al
London EC1N 2JT (GB)

(54) Ornithine carbamoyl transferase gene and its use as a marker gene in a host-vector system for the production of proteins in basidiomycetes

(57) This invention relates to a novel ornithine carbamoyl transferase (OCTase) gene, a recombinant DNA containing the OCTase DNA, a transformation system for a basidiomycete, and production of useful proteins using the system.

More particularly, this invention relates to the OCTase gene of Coriolus hirsutus to afford an efficient host-vector system in basidiomycetes (particularly a white rot fungus such as Coriolus hirsutus (FO 4917)) for the preparation of useful proteins. OCTase is the enzyme to transform ornithine to citrulline in arginine biosynthesis in organisms. The present invention provides the OCTase genes of C. hirsutus, the useful Arg⁻ auxotrophic mutant of C. hirsutus deficient in the OCTase gene, the efficient condition for the preparation of protoplasts and the transformation of the mutant with the cloned OCTase gene and the recombinant DNA's including a promoter, a signal peptide-coding DNA and a protein-coding DNA, the construction of the novel host-vector system of C. hirsutus, and the new method of a large scale preparation of useful proteins using this novel host-vector system with a new recombinant DNA technique.

Furthermore, this invention provides a highly efficient method to produce a useful protein such as lignin peroxidase which is difficult to produce by the conventional method.

EP 0 570 096 A3

European Patent Office

EUROPEAN SEARCH REPORT

Application Number
EP 93 30 1737

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
A	EP-A-0 388 166 (OJI PAPER CO. LTD.) *Example 12 and Claims* ---	1	C12N15/54 C12N15/80 C12N1/15 C12N1/14 C12N15/53 C12N15/62 //(C12N1/15, C12R1:645)
D,X A	MOL. GEN. GENET., vol. 204, 1986 pages 349-354, A. UPSHALL ET AL.; 'Molecular analysis of the argB gene of Aspergillus nidulans' *abstract and Figure 4* ---	3 1	
D,X A	GENE, vol. 60, 1987 pages 255-266, F.P. BUXTON ET AL.; 'Cloning and molecular analysis of the ornithine carbamoyl transferase gene of Aspergillus niger' *abstract, introduction and Figure 4* -----	3 1	
			TECHNICAL FIELDS SEARCHED (Int.Cl.5) C12N

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
MUNICH	30 October 1995	Yeats, S

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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